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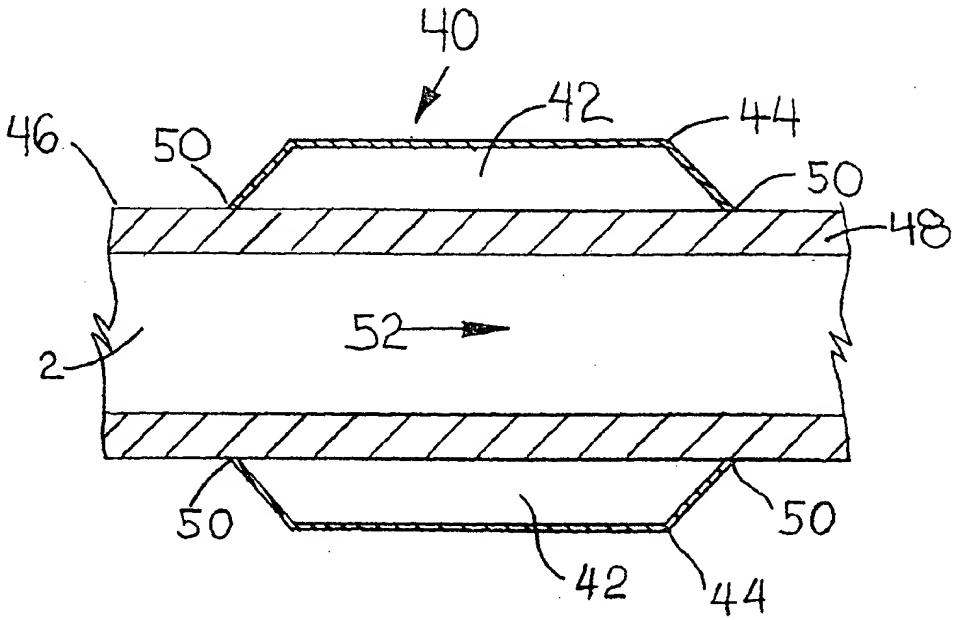

(57) Abstract

The present invention provides materials and methods for preventing stenosis or restenosis of a blood vessel using Vascular Endothelial Growth Factor C (VEGF–C) and/or Vascular Endothelial Growth Factor D (VEGF–D) genes or proteins.

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USE OF VEGF-C OR VEGF-D GENE OR PROTEIN TO PREVENT RESTENOSIS

FIELD OF THE INVENTION

The present invention provides materials and methods to prevent stenosis and restenosis of blood vessels, and relates generally to the field of cardiovascular medicine.

BACKGROUND OF THE INVENTION

Coronary artery disease constitutes a major cause of morbidity and mortality throughout the world, especially in the United States and Europe. Percutaneous transluminal coronary angioplasty (*e.g.*, balloon angioplasty, with our without intracoronary stenting) is now a common and successful therapy for such disease, performed hundreds of thousands of times per year in the United States alone. However, restenosis occurs in as many as one-third to one-half of such revascularization procedures, usually within six months of the angioplasty procedure. The economic cost of restenosis has been estimated at $2 billion annually in the United States alone. [Feldman *et al.*, *Cardiovascular Research, 32*: 194-207 (1996), incorporated herein by reference.] Autopsy and atherectomy studies have identified intimal hyerplasia as the major histologic component of restenotic lesions. [Cerek *et al., Am. J. Cardiol., 68*: 24C-33C (1991).]

Restenosis also remains a clinical concern in angioplasty that is performed in peripheral blood vessels. Likewise, stenosis is a clinical concern following transplantation of blood vessels (e.g., grafted veins and grafted artificial vessels) for cardiac bypass therapy or for treatment of peripheral ischemia or intermittent claudication, for example (e.g., above-knee femoro-popliteal arterial bypass grafts).

Mazur *et al., Texas Heart Institute Journal, 21*; 104-111 (1994) state that restenosis is primarily a response of the artery to the injury caused by percutaneous coronary angioplasty, which disrupts the intimal layer of endothelial cells and underlying

smooth muscle cells of the media. The authors state that multiple growth factors secreted by platelets, endothelial cells, macrophages, and smooth muscle cells are mechanistically involved in the restenosis process, and that proliferation of smooth muscle cells constitutes a critical pathogenetic feature. According to the authors, this smooth muscle cell proliferation has proven refractory to mechanical and pharmacologic therapy. More recently, others have called into question whether smooth muscle cell proliferation is of penultimate importance in restenosis. See Libby, *Circ. Res., 82*: 404-406 (1998).

Narins *et al, Circulation, 97*: 1298-1305 (1998) review the use of intracoronary stents and their benefits and limitations in preventing restenosis. Debbas *et al., American Heart Journal, 133*: 460-468 (1997) discuss stenting within a stent to treat in-stent restenosis.

Chang & Leiden, *Semin. Intervent. Cardiol., 1*: 185-193 (1996), incorporated herein by reference, review somatic gene therapy approaches to treat restenosis. Chang and Leiden teach that replication-deficient adenoviruses comprise a promising and safe vector system for gene therapy directed toward prevention of restenosis, because such viruses can efficiently infect a wide variety of cell types, including vascular smooth muscle cells; such viruses can be produced at high titers (*e.g.*, 10^{10}-10^{12} plaque forming units per milliliter); such viruses can accommodate a transgene insert of, *e.g.*, 7-9 kilobases (kb) in size; such viruses can be delivered percutaneously through standard catheters; and such viruses do not integrate into the host genome. Both Chang & Leiden and Feldman *et al., supra*, also review cytotoxic and cytostatic gene therapy approaches, designed to kill or arrest proliferating vascular smooth muscle cells thought to be responsible for neointimal formations that characterize restenosis.

Riessen & Isner, *J. Am. Coll. Cardiol., 23*:1234-1244 (1994), incorporated by reference, review devices for intravascular drug delivery and vectors for intravascular gene therapy.

Cerek *et al., Am. J. Cardiol., 68*: 24C-33C (1991) suggest prevention of restenosis by inhibiting growth-factor-mediated healing of arterial injury. Potential roles of platelet-derived growth factor (PDGF), thrombospondin, insulin-like growth factor 1 (IGF-1), fibroblast growth factors (FGF's), transforming growth factor alpha (TGF-α) and beta (TGF-β), epidermal growth factor (EGF) are discussed.

Isner & Asahara, International Patent Publication No. WO 98/19712, incorporated herein by reference, suggest treating injured blood vessels and accelerating reendothelialization following angioplasty by isolating a patient's endothelial progenitor cells and re-administering such cells to the patient. The authors suggest that the effectiveness of using an angiogenesis-promoting growth factor, such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF), may be limited by the lack of endothelial cells on which the VEGF or bFGF will exert its effect.

Martin *et al.*, International Patent Publication No. WO 98/20027 suggest the use of VEGF gene or protein to treat or prevent stenosis or restenosis of a blood vessel. The authors suggest that any beneficial effect of VEGF arises from a different mechanism of action than the mechanism underlying an activity of VEGF related to stimulating re-endothelialisation in cases where the endothelium has been damaged.

Callow *et al., Growth Factors, 10*: 223-228 (1994) state that intravenous injection of vascular permeability factor (a.k.a. VEGF) into rabbits that had been subjected to balloon angioplasty-induced endothelial denudation resulted in increased regeneration of endothelium compared to a control. The authors also stated that basic fibroblast growth factor (bFGF) is effective at promoting re-endothelialization, but that such re-endothelialization is accompanied by increases in neointimal lesion size.

Asahara *et al., Circulation, 94*: 3291-3302 (December 15, 1996) state that local, percutaneous catheter delivery of a CMV-human-$VEGF_{165}$ transgene achieved accelerated re-endothelialization in balloon-injured rabbits, and resulted in diminished intimal thickening. In a report by a related group of authors, Van Belle *et al., J. Am. Coll. Cardiol., 29*:1371-1379 (May, 1997) state that stent endothelialization was accelerated by delivery of a CMV-human-$VEGF_{165}$ transgene and was accompanied by attenuation of intimal thickening.

Morishita *et al., J. Atherosclerosis and Thrombosis, 4(3)*: 128-134 (1998) state that hepatocyte growth factor (HGF) has a mitogenic activity on human endothelial cells more potent than VEGF, and hypothesized that HGF gene therapy may have potential therapeutic value for the treatment of cardiovascular diseases such as restenosis after angioplasty. Morishita *et al.* also state that there is little knowledge about growth factors that stimulate only endothelial cells, but not vascular smooth muscle cells.

DeYoung & Dichek, *Circ. Res., 82*: 306-313 (1998) state that VEGF gene delivery does not currently appear destined for application to human coronary restenosis, and that two independent studies suggest that VEGF delivery may actually worsen arterial intimal hyperplasia.

Brown *et al.*, U.S. Patent No. 5,795,898, suggest using an inhibitor of PDGF, FGF, EGF, or VEGF signaling to suppress accelerated atherogenesis involved in restenosis of coronary vessels or other arterial vessels following angioplasty.

The foregoing discussion demonstrates that a long-felt need continues to exist for improvements to angioplasty materials and/or methods, and/or for adjunct therapies, to reduce instances of restenosis.

SUMMARY OF THE INVENTION

The present invention addresses long-felt needs in the field of medicine by providing materials and methods for the prevention of stenosis or restenosis in mammalian blood vessels.

For example, the invention provides a method of treating a mammalian subject to prevent stenosis or restenosis of a blood vessel, comprising the step of administering to a mammalian subject in need of treatment to prevent stenosis or restenosis of a blood vessel a composition comprising a polynucleotide, the polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor C (VEGF-C) polypeptide. In a preferred embodiment, the subject is a human subject.

While it is contemplated that the VEGF-C polynucleotide could be administered purely as a prophylactic treatment to prevent stenosis, it is contemplated in a preferred embodiment that the polynucleotide be administered shortly before, and/or concurrently with, and/or shortly after a percutaneous transluminal coronary angioplasty procedure, for the purpose of preventing restenosis of the subject vessel. In another preferred embodiment, the polynucleotide is administered before, during, and/or shortly after a bypass procedure (*e.g.*, a coronary bypass procedure), to prevent stenosis or restenosis in or near the transplanted (grafted) vessel, especially stenosis at the location of the graft itself. In yet another embodiment, the polynucleotide is administered before, during, or after a vascular transplantation in the vascular periphery that has been

performed to treat peripheral ischemia or intermittent claudication. By prevention of stenosis or restenosis is meant prophylactic treatment to reduce the amount/severity of, and/or substantially eliminate, the stenosis or restenosis that frequently occurs in such surgical procedures. The polynucleotide is included in the composition in an amount and in a form effective to promote expression of a VEGF-C polypeptide in a blood vessel of the mammalian subject, thereby preventing stenosis or restenosis of the blood vessel.

In a preferred embodiment, the mammalian subject is a human subject. For example, the subject is a person suffering from coronary artery disease that has been identified by a cardiologist as a candidate who could benefit from a therapeutic balloon angioplasty (with or without insertion of an intravascular stent) procedure or from a coronary bypass procedure. Practice of methods of the invention in other mammalian subjects, especially mammals that are conventionally used as models for demonstrating therapeutic efficacy in humans (e.g., primate, porcine, canine, or rabbit animals), also is contemplated.

For the practice of methods of the invention, the term "VEGF-C polypeptide" is intended to include any polypeptide that has a VEGF-C or VEGF-C analog amino acid sequence (as defined elsewhere herein in greater detail) and that possesses *in vivo* restenosis-reducing effects of human VEGF-C, which effects are demonstrated herein by way of example in a rabbit model. The term "VEGF-C polynucleotide" is intended to include any polynucleotide (*e.g.*, DNA or RNA, single- or double-stranded) comprising a nucleotide sequence that encodes a VEGF-C polypeptide. Due to the well-known degeneracy of the genetic code, there exist multiple VEGF-C polynucleotide sequences that encode any selected VEGF-C polypeptide.

For treatment of humans, VEGF-C polypeptides with an amino acid sequence of a human VEGF-C are highly preferred, and polynucleotides comprising a nucleotide sequence of a human VEGF-C cDNA are highly preferred. By "human VEGF-C" is meant a polypeptide corresponding to a naturally occurring protein (prepro-protein, partially-processed protein, or fully-processed mature protein) encoded by any allele of the human VEGF-C gene, or a polypeptide comprising a biologically active fragment of a naturally-occurring mature protein. By way of example, a human VEGF-C comprises a continuous portion of the amino acid sequence set forth in SEQ ID NO: 2 sufficient to

permit the polypeptide to bind and stimulate VEGFR-2 and/or VEGFR-3 phosphorylation in cells that express such receptors. A polypeptide comprising amino acids 131-211 of SEQ ID NO: 2 is specifically contemplated. For example, polypeptides having an amino acid sequence comprising a continuous portion of SEQ ID NO: 2, the continuous portion having, as its amino terminus, an amino acid selected from the group consisting of positions 30-131 of SEQ ID NO: 2, and having, as its carboxyl terminus, an amino acid selected from the group consisting of positions 211-419 of SEQ ID NO: 2 are contemplated. As explained elsewhere herein in greater detail, VEGF-C biological activities, especially those mediated through VEGFR-2, increase upon processing of both an amino-terminal and carboxyl-terminal pro-peptide. Thus, an amino terminus selected from the group consisting of positions 102-131 of SEQ ID NO: 2 is preferred, and an amino terminus selected from the group consisting of positions 103-113 of SEQ ID NO: 2 is highly preferred. Likewise, a carboxyl terminus selected from the group consisting of positions 211-227 of SEQ ID NO: 2 is preferred. As stated above, the term "human VEGF-C" also is intended to encompass polypeptides encoded by allelic variants of the human VEGF-C characterized by the sequences set forth in SEQ ID NOs: 1 & 2.

Moreover, since the therapeutic VEGF-C is to be administered as recombinant VEGF-C or indirectly via somatic gene therapy, it is within the skill in the art to make and use analogs of human VEGF-C (and polynucleotides that encode such analogs) wherein one or more amino acids have been added, deleted, or replaced with other amino acids, especially with conservative replacements, and wherein the anti-restenosis biological activity has been retained. Analogs that retain anti-restenosis VEGF-C biological activity are contemplated as VEGF-C polypeptides for use in the present invention. In a preferred embodiment, analogs having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 such modifications and that retain anti-restenosis VEGF-C biological activity are contemplated as VEGF-C polypeptides for use in the present invention. Polynucleotides encoding such analogs are generated using conventional PCR, site-directed mutagenesis, and chemical synthesis techniques.

Also contemplated as VEGF-C polypeptides are non-human mammalian or avian VEGF-C polypeptides and polynucleotides. By "mammalian VEGF-C" is meant a polypeptide corresponding to a naturally occurring protein (prepro-protein, partially-

processed protein, or fully-processed mature protein) encoded by any allele of a VEGF-C gene of any mammal, or a polypeptide comprising a biologically active fragment of a mature protein. The term "mammalian VEGF-C polypeptide" is intended to include analogs of mammalian VEGF-C's that possess the *in vivo* restenosis-reducing effects of the mammalian VEGF-C. The fact that gene therapy using a transgene encoding human VEGF-C is effective to prevent restenosis in a rabbit model is evidence of the inter-species therapeutic efficacy of VEGF-C proteins.

Irrespective of which VEGF-C polypeptide is chosen, the VEGF-C polynucleotide preferably comprises a nucleotide sequence encoding a secretory signal peptide fused in-frame with the VEGF-C polypeptide sequence. The secretory signal peptide directs secretion of the VEGF-C polypeptide by the cells that express the polynucleotide, and is cleaved by the cell from the secreted VEGF-C polypeptide. For example, the VEGF-C polynucleotide could encode the complete prepro-VEGF-C sequence set forth in SEQ ID NO: 2; or could encode the VEGF-C signal peptide fused in-frame to a sequence encoding a fully-processed VEGF-C (*e.g.*, amino acids 103-227 of SEQ ID NO: 2) or VEGF-C analog. Moreover, there is no requirement that the signal peptide be derived from VEGF-C. The signal peptide sequence can be that of another secreted protein, or can be a completely synthetic signal sequence effective to direct secretion in cells of the mammalian subject.

In one embodiment, the VEGF-C polynucleotide of the invention comprises a nucleotide sequence that will hybridize to a polynucleotide that is complementary to the human VEGF cDNA sequence specified in SEQ ID NO: 1 under the following exemplary stringent hybridization conditions: hybridization at 42°C in 50% formamide, 5X SSC, 20 mM $Na•PO_4$, pH 6.8; and washing in 1X SSC at 55°C for 30 minutes; and wherein the nucleotide sequence encodes a polypeptide that binds and stimulates human VEGFR-2 and/or VEGFR-3. It is understood that variation in these exemplary conditions occur based on the length and GC nucleotide content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining appropriate hybridization conditions. *See* Sambrook *et al., Molecular Cloning: A Laboratory Manual* (Second ed., Cold Spring Harbor Laboratory Press, 1989) §§ 9.47-9.51.

In preferred embodiments, the VEGF-C polynucleotide further comprises additional sequences to facilitate the VEGF-C gene therapy. In one embodiment, a "naked" VEGF-C transgene (i.e., a transgene without a viral, liposomal, or other vector to facilitate transfection) is employed for gene therapy. In this embodiment, the VEGF-C polynucleotide preferably comprises a suitable promoter and/or enhancer sequence (*e.g.*, cytomegalovirus promoter/enhancer [Lehner *et al., J. Clin. Microbiol., 29*:2494-2502 (1991); Boshart *et al., Cell, 41*:521-530 (1985)]; Rous sarcoma virus promoter [Davis *et al., Hum. Gene Ther.*, 4:151 (1993)]; Tie promoter [Korhonen *et al., Blood, 86(5)*: 1828-1835 (1995)]; or simian virus 40 promoter) for expression in the target mammalian cells, the promoter being operatively linked upstream (i.e., 5') of the VEGF-C coding sequence. The VEGF-C polynucleotide also preferably further includes a suitable polyadenylation sequence (*e.g.*, the SV40 or human growth hormone gene polyadenylation sequence) operably linked downstream (i.e., 3') of the VEGF-C coding sequence. The polynucleotide may further optionally comprise sequences whose only intended function is to facilitate large-scale production of the vector, *e.g.*, in bacteria, such as a bacterial origin of replication and a sequence encoding a selectable marker. However, in a preferred embodiment, such extraneous sequences are at least partially cleaved off prior to administration to humans according to methods of the invention. One can manufacture and administer such polynucleotides to achieve successful gene therapy using procedures that have been described in the literature for other transgenes. See, *e.g.*, Isner *et al., Circulation, 91*: 2687-2692 (1995); and Isner *et al., Human Gene Therapy, 7*: 989-1011 (1996); incorporated herein by reference in the entirety.

Any suitable vector may be used to introduce the VEGF-C transgene into the host. Exemplary vectors that have been described in the literature include replication-deficient retroviral vectors, including but not limited to lentivirus vectors [Kim *et al., J. Virol., 72(1)*: 811-816 (1998); Kingsman & Johnson, *Scrip Magazine*, October, 1998, pp. 43-46.]; adeno-associated viral vectors [Gnatenko *et al., J. Investig. Med., 45*: 87-98 (1997)]; adenoviral vectors [See, *e.g.*, U.S. Patent No. 5,792,453; Quantin *et al., Proc. Natl. Acad. Sci. USA, 89*: 2581-2584 (1992); Stratford-Perricadet *et al., J. Clin. Invest., 90*: 626-630 (1992); and Rosenfeld *et al., Cell, 68*: 143-155 (1992)]; Lipofectin-mediated gene transfer (BRL); liposomal vectors [See, *e.g.*, U.S. Patent No. 5,631,237 (Liposomes

comprising Sendai virus proteins)] ; and combinations thereof. All of the foregoing documents are incorporated herein by reference in the entirety. Replication-deficient adenoviral vectors constitute a preferred embodiment.

In embodiments employing a viral vector, preferred polynucleotides still include a suitable promoter and polyadenylation sequence as described above. Moreover, it will be readily apparent that, in these embodiments, the polynucleotide further includes vector polynucleotide sequences (*e.g.*, adenoviral polynucleotide sequences) operably connected to the sequence encoding a VEGF-C polypeptide.

Thus, in one embodiment the composition to be administered comprises a vector, wherein the vector comprises the VEGF-C polynucleotide. In a preferred embodiment, the vector is an adenovirus vector. In a highly preferred embodiment, the adenovirus vector is replication-deficient, i.e., it cannot replicate in the mammalian subject due to deletion of essential viral-replication sequences from the adenoviral genome. For example, the inventors contemplate a method wherein the vector comprises a replication-deficient adenovirus, the adenovirus comprising the VEGF-C polynucleotide operably connected to a promoter and flanked on either end by adenoviral polynucleotide sequences.

The composition to be administered according to methods of the invention preferably comprises (in addition to the polynucleotide or vector) a pharmaceutically-acceptable carrier solution such as water, saline, phosphate-buffered saline, glucose, or other carriers conventionally used to deliver therapeutics intravascularly. Multi-gene therapy is also contemplated, in which case the composition optionally comprises both the VEGF-C polynucleotide/vector and another polynucleotide/vector selected to prevent restenosis. Exemplary candidate genes/vectors for co-transfection with VEGF-C transgenes are described in the literature cited above, including genes encoding cytotoxic factors, cytostatic factors, endothelial growth factors, and smooth muscle cell growth/migration inhibitors. As described in greater detail below, a VEGF-D transgene is a preferred candidate for co-administration with the VEGF-C transgene. Co-administration of a VEGF transgene also is specifically contemplated.

The "administering" that is performed according to the present method may be performed using any medically-accepted means for introducing a therapeutic directly or

indirectly into the vasculature of a mammalian subject, including but not limited to injections; oral ingestion; intranasal or topical administration; and the like. In a preferred embodiment, administration of the composition comprising the VEGF-C polynucleotide is performed intravascularly, such as by intravenous, intra-arterial, or intracoronary arterial injection.

In a highly preferred embodiment, the composition is administered locally, *e.g.*, to the site of angioplasty or bypass. For example, the administering comprises a catheter-mediated transfer of the transgene-containing composition into a blood vessel of the mammalian subject, especially into a coronary artery of the mammalian subject. Exemplary materials and methods for local delivery are reviewed in Lincoff *et al.*, *Circulation, 90*: 2070-2084 (1994); and Wilensky *et al.*, *Trends Cardiovasc. Med., 3:*163-170 (1993), both incorporated herein by reference. For example, the composition is administered using infusion-perfusion balloon catheters (preferably mircroporous balloon catheters) such as those that have been described in the literature for intracoronary drug infusions. See, *e.g.*, U.S. Patent No. 5,713,860 (Intravascular Catheter with Infusion Array); U.S. Patent No. 5,087,244; U.S. Patent No. 5,653,689; and Wolinsky *et al.*, *J. Am. Coll. Cardiol., 15*: 475-481 (1990) (Wolinsky Infusion Catheter); and Lambert *et al.*, *Coron. Artery Dis., 4*: 469-475 (1993), all of which are incorporated herein by reference in their entirety. Use of such catheters for site-directed somatic cell gene therapy is described, *e.g.*, in Mazur *et al.*, *Texas Heart Institute Journal, 21*; 104-111 (1994), incorporated herein by reference. In an embodiment where the VEGF-C transgene is administered in an adenovirus vector, the vector is preferably administered in a pharmaceutically acceptable carrier at a titer of 10^7-10^{13} viral particles, and more preferably at a titer of 10^9-10^{11} viral particles. The adenoviral vector composition preferably is infused over a period of 15 seconds to 30 minutes, more preferably 1 to 10 minutes.

For example, in patients with angina pectoris due to a single or multiple lesions in coronary arteries and for whom PTCA is prescribed on the basis of primary coronary angiogram findings, an exemplary protocol involves performing PTCA through a 7F guiding catheter according to standard clinical practice using the femoral approach. If an optimal result is not achieved with PTCA alone, then an endovascular stent also is

implanted. (A nonoptimal result is defined as residual stenosis of > 30 % of the luminal diameter according to a visual estimate, and B or C type dissection.) Arterial gene transfer at the site of balloon dilatation is performed with a replication-deficient adenoviral VEGF-C vector immediately after the angioplasty, but before stent implantation, using an infusion-perfusion balloon catheter. The size of the catheter will be selected to match the diameter of the artery as measured from the angiogram, varying, *e.g.*, from 3.0 to 3.5F in diameter. The balloon is inflated to the optimal pressure and gene transfer is performed during a 10 minute infusion at the rate of 0.5 ml/min with virus titer of 1.15×10^{10}.

In another embodiment, intravascular administration with a gel-coated catheter is contemplated, as has been described in the literature to introduce other transgenes. See, *e.g.*, U.S. Patent No. 5,674,192 (Catheter coated with tenaciously-adhered swellable hydrogel polymer); Riessen *et al.*, *Human Gene Therapy, 4*: 749-758 (1993); and Steg *et al.*, *Circulation, 96*: 408-411 (1997) and *90*: 1648-1656 (1994); all incorporated herein by reference. Briefly, DNA in solution (*e.g.*, the VEGF-C polynucleotide) is applied one or more times *ex vivo* to the surface of an inflated angioplasty catheter balloon coated with a hydrogel polymer (e.g., Slider with Hydroplus, Mansfield Boston Scientific Corp., Watertown, MA). The Hydroplus coating is a hydrophilic polyacrylic acid polymer that is cross-linked to the balloon to form a high molecular weight hydrogel tightly adhered to the balloon. The DNA covered hydrogel is permitted to dry before deflating the balloon. Re-inflation of the balloon intravascularly, during an angioplasty procedure, causes the transfer of the DNA to the vessel wall.

In yet another embodiment, an expandable elastic membrane or similar structure mounted to or integral with a balloon angioplasty catheter or stent is employed to deliver the VEGF-C transgene. See, *e.g.*, U.S. Patent Nos. 5,707,385, 5,697,967, 5,700,286, 5,800,507, and 5,776,184, all incorporated by reference herein.

In another variation, the composition containing the VEGF-C transgene is administered extravascularly, e.g., using a device to surround or encapsulate a portion of vessel. See, e.g., International Patent Publication WO 98/20027, incorporated herein by reference, describing a collar that is placed around the outside of an artery (*e.g.*, during a bypass procedure) to deliver a transgene to the arterial wall via a plasmid or liposome vector.

In still another variation, endothelial cells or endothelial progenitor cells are transfected *ex vivo* with the VEGF-C transgene, and the transfected cells as administered to the mammalian subject. Exemplary procedures for seeding a vascular graft with genetically modified endothelial cells are described in U.S. Patent No. 5,785,965, incorporated herein by reference.

If the mammalian subject is receiving a vascular graft, the VEGF-C transgene-containing composition may be directly applied to the isolated vessel segment prior to its being grafted *in vivo*.

In another aspect, the invention provides a method of treating a mammalian subject to prevent stenosis or restenosis of a blood vessel, comprising the step of administering to a mammalian subject in need of treatment to prevent stenosis or restenosis of a blood vessel a composition comprising a VEGF-C polypeptide, in an amount effective to prevent stenosis or restenosis of the blood vessel. In a preferred embodiment, the administering comprises implanting an intravascular stent in the mammalian subject, where the stent is coated or impregnated with the composition. Exemplary materials for constructing a drug-coated or drug-impregnated stent are described in literature cited above and reviewed in Lincoff *et al., Circulation, 90*: 2070-2084 (1994). In another preferred embodiment, the composition comprises microparticles composed of biodegradable polymers such as PGLA, non-degradable polymers, or biological polymers (e.g., starch) which particles encapsulate or are impregnated by the VEGF-C polypeptide. Such particles are delivered to the intravascular wall using, e.g., an infusion angioplasty catheter. Other techniques for achieving locally sustained drug delivery are reviewed in Wilensky *et al., Trends Caridovasc. Med., 3*:163-170 (1993), incorporated herein by reference.

Administration via one or more intravenous injections subsequent to the angioplasty or bypass procedure also is contemplated. Localization of the VEGF-C polypeptides to the site of the procedure occurs due to expression of VEGF-C receptors on proliferating endothelial cells. Localization is further facilitated by recombinantly expressing the VEGF-C as a fusion polypeptide (*e.g.*, fused to an apolipoprotein B-100 oligopeptide as described in Shih *et al., Proc. Nat'l. Acad. Sci. USA, 87*:1436-1440

(1990). Co-administration of VEGF-C polynucleotides and VEGF-C polypeptides also is contemplated.

In yet another embodiment, the invention provides the use of a VEGF-C polynucleotide or VEGF-C polypeptide for the manufacture of a medicament for the treatment or prevention of stenosis or restenosis of a blood vessel.

In still another embodiment, the invention provides a method of treating a mammalian subject to prevent stenosis or restenosis of a blood vessel, comprising the step of administering to a mammalian subject in need of treatment to prevent stenosis or restenosis of a blood vessel a composition comprising a polynucleotide, the polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor D (VEGF-D) polypeptide. Such methods are practiced essentially as described herein with respect to VEGF-C-encoding polynucleotides, except that polynucleotides encoding VEGF-D are employed. A detailed description of the human VEGF-D gene and protein are provided in Achen, *et al., Proc. Nat'l Acad. Sci. U.S.A., 95(2)*: 548-553 (1998); International Patent Publication No. WO 98/07832, published 26 February 1998; and in Genbank Accession No. AJ000185, all incorporated herein by reference. A cDNA and deduced amino acid sequence for prepro-VEGF-D is set forth herein in SEQ ID NOs: 3 and 4. Of course, due to the well-known degeneracy of the genetic code, there exist multiple VEGF-D encoding polynucleotide sequences, any of which may be employed according to the methods taught herein.

As described herein in detail with respect to VEGF-C, the use of polynucleotides that encode VEGF-D fragments, VEGF-D analogs, VEGF-D allelic and interspecies variants, and the like which possess *in vivo* anti-restenosis effects of human VEGF-D are all contemplated as being encompassed by the present invention.

In yet another embodiment, the invention provides a method of treating a mammalian subject to prevent stenosis or restenosis of a blood vessel, comprising the step of administering to a mammalian subject in need of treatment to prevent stenosis or restenosis of a blood vessel a composition comprising a VEGF-D polypeptide, in an amount effective to prevent stenosis or restenosis of the blood vessel. Such methods are practiced essentially as described herein with respect to VEGF-C polypeptides.

In a related aspect, the invention provides materials and devices for practice of the above-described methods.

For example, the polynucleotides, polypeptides, vectors, compositions, and the like that are described for use in methods of the invention are themselves intended as aspects of the invention.

Likewise, the invention also provides surgical devices that are used to treat circulatory disorders, such as intravascular (endovascular) stents, balloon catheters, infusion-perfusion catheters, extravascular collars, elastomeric membranes, and the like, which have been improved by coating with, impregnating with, adhering to, or encapsulating within the device a composition comprising a VEGF-C polynucleotide, a VEGF-C polypeptide, a VEGF-D polynucleotide, and/or a VEGF-D polypeptide.

For example, in one embodiment, the invention provides an endovascular stent characterized by an improvement wherein the stent is coated or impregnated with a composition, the comprising at least one anti-restenosis agent selected from the group consisting of VEGF-C polynucleotides, VEGF-C polypeptides, VEGF-D polynucleotides, and VEGF-D polypeptides. Exemplary stents that may be improved in this manner are described and depicted in U.S. Patent Nos. 5,800,507 and 5,697,967 (Medtronic, Inc., describing an intraluminal stent comprising fibrin and an elutable drug capable of providing a treatment of restenosis); U.S. Patent No. 5,776,184 (Medtronic, Inc., describing a stent with a porous coating comprising a polymer and a therapeutic substance in a solid or solid/solution with the polymer); U.S. Patent No. 5,799,384 (Medtronic, Inc., describing a flexible, cylindrical, metal stent having a biocompatible polymeric surface to contact a body lumen); U.S. Patent Nos. 5,824,048 and 5,679,400; and U.S. Patent No. 5,779,729; all of which are specifically incorporated herein by reference in the entirety. Implantation of such stents during conventional angioplasty techniques will result in less restenosis than implantation of conventional stents. In this sense, the biocompatibility of the stent is improved.

In another embodiment, the invention provides an extravascular collar for delivery of a therapeutic agent to a blood vessel, characterized by an improvement wherein the collar is coated with or impregnated with or encapsulates a composition, the comprising at least one anti-restenosis agent selected from the group consisting of VEGF-

C polynucleotides, VEGF-C polypeptides, VEGF-D polynucleotides, and VEGF-D polypeptides. An exemplary collar to be improved in this manner is described and depicted in International Patent Publication WO 98/20027 (Eurogene, Ltd., collar comprising a body adopted to provide a seal around a vessel and to define a reservoir for holding an anti-restenosis pharmaceutical formulation), incorporated herein by reference.

In yet another embodiment, the invention provides a polymer film for wrapping a stent, characterized by an improvement wherein the film is coated with or impregnated with a composition, the comprising at least one anti-restenosis agent selected from the group consisting of VEGF-C polynucleotides, VEGF-C polypeptides, VEGF-D polynucleotides, and VEGF-D polypeptides. An exemplary film to be improved in this manner is described and depicted in U.S. Patent Nos. 5,700,286 and 5,707,385 (Advanced Cardiovascular Systems, Inc., sheaths of bioabsorbable polymeric material coated or impregnated with a restenosis-preventing therapeutic agent and attachable to an endovascular stent).

Similarly, the invention includes kits which comprise compounds or compositions of the invention packaged in a manner which facilitates their use to practice methods of the invention. In a simplest embodiment, such a kit includes a compound or composition described herein as useful for practice of the invention (e.g., VEGF-C or VEGF-D polynucleotides or polypeptides), packaged in a container such as a sealed bottle or vessel, with a label affixed to the container or included in the package that describes use of the compound or composition to practice the method of the invention. Preferably, the compound or composition is packaged in a unit dosage form. In another embodiment, a kit of the invention includes both a VEGF-C or VEGF-D polynucleotide or polypeptide composition packaged together with a physical device useful for implementing methods of the invention, such as a stent, a catheter, an extravascular collar, a polymer film, or the like. In another embodiment, a kit of the invention includes both a VEGF-C or VEGF-D polynucleotide or polypeptide composition packaged together with a hydrogel polymer, or microparticle polymers, or other carriers described herein as useful for delivery of the VEGF-C/VEGF-D to the patient.

Additional features and variations of the invention will be apparent to those skilled in the art from the entirety of this application, and all such features are intended as aspects of the invention.

Likewise, features of the invention described herein can be re-combined into additional embodiments that also are intended as aspects of the invention, irrespective of whether the combination of features is specifically mentioned above as an aspect or embodiment of the invention. Also, only such limitations which are described herein as critical to the invention should be viewed as such; variations of the invention lacking limitations which have not been described herein as critical are intended as aspects of the invention.

In addition to the foregoing, the invention includes, as an additional aspect, all embodiments of the invention narrower in scope in any way than the variations specifically mentioned above. Although the applicant(s) invented the full scope of the claims appended hereto, the claims appended hereto are not intended to encompass within their scope the prior art work of others. Therefore, in the event that statutory prior art within the scope of a claim is brought to the attention of the applicants by a Patent Office or other entity or individual, the applicant(s) reserve the right to exercise amendment rights under applicable patent laws to redefine the subject matter of such a claim to specifically exclude such statutory prior art or obvious variations of statutory prior art from the scope of such a claim. Variations of the invention defined by such amended claims also are intended as aspects of the invention.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 depicts a cross-section of a blood vessel into which a drug delivery balloon catheter including a protective sheath has been inserted, the protective sheath serving to cover the balloon during insertion and positioning.

Figure 2A depicts a perspective view of an expandable membrane having two layers that are spaced apart, prior to joining edges of the layers to each other.

Figure 2B depicts a perspective view of the membrane of Figure 2A that has been rolled into a tube and had opposite edges adjoined.

Figures 3A and 3B depict, in perspective (3A) and longitudinal cross-section (3B), schematic views of an extravascular collar surrounding a portion of a blood vessel.

Figure 4A depicts in cross-section a wire coated with a polymer or gel that can include (e.g., be impregnated with) a therapeutic composition.

Figure 4B depicts a perspective view of an intravascular stent formed from the wire of Figure 4A.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that when a gene encoding human Vascular Endothelial Growth Factor C (VEGF-C) is administered to a mammal that has suffered a vascular trauma, such as the trauma that can occur during conventional balloon angioplasty procedures, restenosis of the injured vessel is reduced or eliminated. An *in vivo* controlled experiment demonstrating the efficacy of a VEGF-C transgene to prevent restenosis is described in detail in Example 1. Example 2 provides a side-by-side comparative study demonstrating that the anti-restenosis effects of VEGF-C appear superior to the anti-restenosis effects of VEGF administered in a comparable manner.

The growth factor named Vascular Endothelial Growth Factor C (VEGF-C), as well as native human, non-human mammalian, and avian polynucleotide sequences encoding VEGF-C, and VEGF-C variants and analogs, have been described in detail in International Patent Application Number PCT/US98/01973, filed 02 February 1998 and published on 06 August 1998 as International Publication Number WO 98/33917; in Joukov *et al., J. Biol. Chem., 273(12)*: 6599-6602 (1998); and in Joukov *et al., EMBO J., 16(13)*: 3898-3911 (1997), all of which are incorporated herein by reference in the entirety. As explained therein in detail, human VEGF-C is initially produced in human cells as a prepro-VEGF-C polypeptide of 419 amino acids. A cDNA and deduced amino acid sequence for human prepro-VEGF-C are set forth in SEQ ID NOs: 1 and 2, respectively, and a cDNA encoding human VEGF-C has been deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209 (USA), pursuant to the provisions of the Budapest Treaty (Deposit date of 24 July 1995 and ATCC Accession Number 97231). VEGF-C sequences from other species also have

been reported. See Genbank Accession Nos. MMU73620 (Mus musculus); and CCY15837 (Coturnix coturnix) for example, incorporated herein by reference.

The prepro-VEGF-C polypeptide is processed in multiple stages to produce a mature and most active VEGF-C polypeptide of about 21-23 kD (as assessed by SDS-PAGE under reducing conditions). Such processing includes cleavage of a signal peptide (SEQ ID NO: 2, residues 1-31); cleavage of a carboxyl-terminal peptide (corresponding approximately to amino acids 228-419 of SEQ ID NO: 2 and having a pattern of spaced cysteine residues reminiscent of a Balbiani ring 3 protein (BR3P) sequence [Dignam *et al.*, *Gene, 88:*133-40 (1990); Paulsson *et al., J. Mol. Biol., 211:*331-49 (1990)]) to produce a partially-processed form of about 29 kD; and cleavage (apparently extracellularly) of an amino-terminal peptide (corresponding approximately to amino acids 32-103 of SEQ ID NO: 2) to produced a fully-processed mature form of about 21-23 kD. Experimental evidence demonstrates that partially-processed forms of VEGF-C (*e.g.*, the 29 kD form) are able to bind the Flt4 (VEGFR-3) receptor, whereas high affinity binding to VEGFR-2 occurs only with the fully processed forms of VEGF-C. It appears that VEGF-C polypeptides naturally associate as non-disulfide linked dimers.

Moreover, it has been demonstrated that amino acids 103-227 of SEQ ID NO: 2 are not all critical for maintaining VEGF-C functions. A polypeptide consisting of amino acids 113-213 (and lacking residues 103-112 and 214-227) of SEQ ID NO: 2 retains the ability to bind and stimulate VEGF-C receptors, and it is expected that a polypeptide spanning from about residue 131 to about residue 211 will retain VEGF-C biological activity. The cysteine residue at position 156 has been shown to be important for VEGFR-2 binding ability. However, VEGF-C ΔC_{156} polypeptides (i.e., analogs that lack this cysteine due to deletion or substitution) remain potent activators of VEGFR-3. If the anti-restenosis effects of VEGF-C are mediated through VEGFR-3, then use of VEGF-C ΔC_{156} polypeptides (and polynucleotides encoding them) is expected to provide anti-restenosis efficacy while minimizing VEGFR-2-mediated side-effects. The cysteine at position 165 of SEQ ID NO: 2 is essential for binding either receptor, whereas analogs lacking the cysteines at positions 83 or 137 compete with native VEGF-C for binding with both receptors and stimulate both receptors.

An alignment of human VEGF-C with VEGF-C from other species (performed using any generally accepted alignment algorithm) suggests additional residues wherein modifications can be introduced (*e.g.*, insertions, substitutions, and/or deletions) without destroying VEGF-C biological activity. Any position at which aligned VEGF-C polypeptides of two or more species have different amino acids, especially different amino acids with side chains of different chemical character, is a likely position susceptible to modification without concomitant elimination of function. An exemplary alignment of human, murine, and quail VEGF-C is set forth in Figure 5 of PCT/US98/01973.

Apart from the foregoing considerations, it will be understood that innumerable conservative amino acid substitutions can be performed to a wildtype VEGF-C sequence which are likely to result in a polypeptide that retains VEGF-C biological activities, especially if the number of such substitutions is small. By "conservative amino acid substitution" is meant substitution of an amino acid with an amino acid having a side chain of a similar chemical character. Similar amino acids for making conservative substitutions include those having an acidic side chain (glutamic acid, aspartic acid); a basic side chain (arginine, lysine, histidine); a polar amide side chain (glutamine, asparagine); a hydrophobic, aliphatic side chain (leucine, isoleucine, valine, alanine, glycine); an aromatic side chain (phenylalanine, tryptophan, tyrosine); a small side chain (glycine, alanine, serine, threonine, methionine); or an aliphatic hydroxyl side chain (serine, threonine). Addition or deletion of one or a few internal amino acids without destroying VEGF-C biological activities also is contemplated.

Without intending to be limited to a particular theory, the mechanism behind the efficacy of VEGF-C in preventing restenosis is believed to relate to the ability of VEGF-C to stimulate re-endothelialization of the injured vessel (and/or of the intravascular stent) without significant concomitant stimulation of smooth muscle proliferation in the vessel. VEGF-C also may inhibit smooth muscle cell proliferation. Accordingly, candidate VEGF-C analog polypeptides can be rapidly screened first for their ability to bind and stimulate autophosphorylation of known VEGF-C receptors (VEGFR-2 and VEGFR-3). Polypeptides that stimulate one or both known receptors are rapidly re-screened *in vitro* for their mitogenic and/or chemotactic activity against cultured capillary or arterial endothelial cells (*e.g.*, as described in WO 98/33917). Polypeptides with

mitogenic and/or chemotactic activity are then screened *in vivo* as described herein for the ability to prevent restenosis. In this way, variants (analogs) of naturally occurring VEGF-C proteins are rapidly screened to determine whether or not the variants have the requisite biological activity to constitute "VEGF-C polypeptides" for use in the present invention.

The growth factor named Vascular Endothelial Growth Factor D (VEGF-D), as well as human sequences encoding VEGF-D, and VEGF-D variants and analogs, have been described in detail in International Patent Application Number PCT/US97/14696, filed 21 August 1997 and published on 26 February 1998 as International Publication Number WO 98/07832; and in Achen, *et al., Proc. Nat'l Acad. Sci. U.S.A., 95(2)*: 548-553 (1998), both incorporated herein by reference in the entirety. As explained therein in detail, human VEGF-D is initially produced in human cells as a prepro-VEGF-D polypeptide of 354 amino acids. A cDNA and deduced amino acid sequence for human prepro-VEGF-D are set forth in SEQ ID NOs: 3 and 4, respectively. VEGF-D sequences from other species also have been reported. See Genbank Accession Nos. D89628 (Mus musculus); and AF014827 (Rattus norvegicus), for example, incorporated herein by reference.

The prepro-VEGF-D polypeptide has a putative signal peptide of 21 amino acids and is apparently proteolytically processed in a manner analogous to the processing of prepro-VEGF-C. A "recombinantly matured" VEGF-D lacking residues 1-92 and 202-354 of SEQ ID NO: 4 retains the ability to activate receptors VEGFR-2 and VEGFR-3, and appears to associate as non-covalently linked dimers. Thus, preferred VEGF-D polynucleotides include those polynucleotides that comprise a nucleotide sequence encoding amino acids 93-201 of SEQ ID NO: 4. The guidance provided above for introducing function-preserving modifications into VEGF-C polypeptides is also suitable for introducing function-preserving modifications into VEGF-D polypeptides.

A therapeutic or prophylactic treatment of restenosis provided by the present invention involves administering to a mammalian subjection such as a human a composition comprising a VEGF-C or VEGF-D polynucleotide or polypeptide or combination thereof (sometimes generically referred to herein as a "VEGF-C or VEGF-D therapeutic agent").

The "administering" may be performed using any medically-accepted means for introducing a therapeutic directly or indirectly into the vasculature of a mammalian subject, including but not limited to injections; oral ingestion; intranasal or topical administration; and the like. In a preferred embodiment, administration of the composition comprising the VEGF-C or VEGF-D polynucleotide or polypeptide composition is performed intravascularly, such as by intravenous, intra-arterial, or intracoronary arterial injection.

In a highly preferred embodiment, the composition is administered locally, *e.g.*, to the site of angioplasty or bypass. For example, the administering comprises a catheter-mediated transfer of the therapeutic composition into a blood vessel of the mammalian subject, especially into a coronary artery of the mammalian subject. Exemplary materials and methods for local delivery are reviewed in Lincoff *et al.*, *Circulation, 90*: 2070-2084 (1994); and Wilensky *et al., Trends Cardiovasc. Med., 3:*163-170 (1993), both incorporated herein by reference. For example, the composition is administered using infusion-perfusion balloon catheters (preferably mircroporous balloon catheters) such as those that have been described in the literature for intracoronary drug infusions. See, *e.g.*, U.S. Patent No. 5,713,860 (Intravascular Catheter with Infusion Array); U.S. Patent No. 5,087,244; U.S. Patent No. 5,653,689; and Wolinsky *et al., J. Am. Coll. Cardiol., 15*: 475-481 (1990) (Wolinsky Infusion Catheter); and Lambert *et al., Coron. Artery Dis., 4*: 469-475 (1993), all of which are incorporated herein by reference in their entirety. Use of such catheters for site-directed somatic cell gene therapy is described, *e.g.*, in Mazur *et al., Texas Heart Institute Journal, 21*; 104-111 (1994), incorporated herein by reference.

For example, in patients with angina pectoris due to a single or multiple lesions in coronary arteries and for whom PTCA is prescribed on the basis of primary coronary angiogram findings, an exemplary protocol involves performing PTCA through a 7F guiding catheter according to standard clinical practice using the femoral approach. If an optimal result is not achieved with PTCA alone, then an endovascular stent also is implanted. (A nonoptimal result is defined as residual stenosis of > 30 % of the luminal diameter according to a visual estimate, and B or C type dissection.) Arterial gene transfer at the site of balloon dilatation is performed immediately after the angioplasty, but before

stent implantation, using an infusion-perfusion balloon catheter. The size of the catheter will be selected to match the diameter of the artery as measured from the angiogram, varying, *e.g.*, from 3.0 to 3.5F in diameter. The balloon is inflated to the optimal pressure and gene transfer is performed during a 10 minute infusion at the rate of 0.5 ml/min with virus titer of 1.15×10^{10}.

In another embodiment, intravascular administration with a gel-coated catheter is contemplated, as has been described in the literature to introduce other transgenes. See, *e.g.*, U.S. Patent No. 5,674,192 (Catheter coated with tenaciously-adhered swellable hydrogel polymer); Riessen *et al.*, *Human Gene Therapy, 4*: 749-758 (1993); and Steg *et al.*, *Circulation, 96*: 408-411 (1997) and *90*: 1648-1656 (1994); all incorporated herein by reference. As shown in Figure 1, a catheter **10** is provided to which an inflatable baloon **12** is attached at a distal end. The balloon includes a swellable hydrogel polymer coating **14** capable of absorbing a solution comprising a therapeutic VEGF-C or VEGF-D therapeutic agent. Briefly, DNA in solution (*e.g.*, the VEGF-C or VEGF-D polynucleotide) is applied one or more times *ex vivo* to the surface of an inflated angioplasty catheter balloon coated with a hydrogel polymer (e.g., Slider with Hydroplus, Mansfield Boston Scientific Corp., Watertown, MA). The Hydroplus coating is a hydrophilic polyacrylic acid polymer that is cross-linked to the balloon to form a high molecular weight hydrogel tightly adhered to the balloon. The DNA covered hydrogel is permitted to dry before deflating the balloon. Re-inflation of the balloon intravascularly, during an angioplasty procedure, causes the transfer of the DNA to the vessel wall. Thus, referring again to Figure 1, the catheter with attached, coated balloon is inserted into the lumen **16** of a blood vessel **18** while covered by a protective sheath **20** to minimize exposure of the coated balloon to the blood prior to placement at the site of an occlusion **22**. When the instrument has been positioned at the treatment region, the protective sheath is drawn back or the catheter is moved forward to expose the balloon, which is inflated to compress the balloon (and thus the coating) into the vessel wall, causing transfer of the VEGF-C or VEGF-D therapeutic agent to the tissue, in a manner analogous to squeezing liquid from a compressed sponge or transferring wet paint to a surface by contact.

In yet another embodiment, an expandable elastic membrane, film, or similar structure, mounted to or integral with a balloon angioplasty catheter or stent, is employed to deliver the VEGF-C or VEGF-D therapeutic agent. See, *e.g.*, U.S. Patent Nos. 5,707,385, 5,697,967, 5,700,286, 5,800,507, and 5,776,184, all incorporated by reference herein. As shown in Figures 2A-2B, a single layer **30** or multi-layer **30, 32** sheet of elastic membrane material (Fig. 2A) is formed into a tubular structure **34** (Fig. 2B), *e.g.*, by bringing together and adhering opposite edges of the sheet(s), e.g., in an overlapping or a abutting relationship. In this manner the elastomeric material may be wrapped around a catheter balloon or stent. A therapeutic VEGF-C or VEGF-D composition is combined with the membrane using any suitable means, including injection molding, coating, diffusion, and absorption techniques. In the multilayer embodiment depicted in the Figures, the edges of the two layers may be joined to form a fluid-tight seal. In a preferred embodiment, one layer of material is first processed by stretching the material and introducing a plurality of microscopic holes or slits **36**. After the layers have been joined together, the sheet can be stretched and injected with the therapeutic VEGF-C/D composition through one of the holes or slits to fill the cavity that exists between the layers. The sheet is then relaxed, causing the holes to close and sealing the therapeutic composition between the layers until such time as the sheet is again stretched. This occurs, for example, at the time that an endovascular stent or balloon covered by the sheet is expanded within the lumen of a stenosed blood vessel. The expanding stent or balloon presses radially outward against the inner surface **38** of the tubular sheet covering, thus stretching the sheet, opening the holes, and delivering the therapeutic agent to the walls of the vessel.

In another variation, the composition containing the VEGF-C or VEGF-D therapeutic is administered extravascularly, e.g., using a device to surround or encapsulate a portion of vessel. See, e.g., International Patent Publication WO 98/20027, incorporated herein by reference, describing a collar that is placed around the outside of an artery (*e.g.*, during a bypass procedure) to deliver a transgene to the arterial wall via a plasmid or liposome vector. As shown in Figures 3A and 3B, an extravascular collar **40** including a void space **42** defined by a wall **44** formed, e.g., of a biodegradable or biocompatible material. The collar touches the outer wall **46** of a blood vessel **48** at the collar's outer

extremities 50. Blood 52 flows through the lumen of the blood vessel. A longitudinal slit 54 in the flexible collar permits the collar to be deformed and placed around the vessel and then sealed using a conventional tissue glue, such as a thrombin glue.

In still another variation, endothelial cells or endothelial progenitor cells are transfected *ex vivo* with the VEGF-C a VEGF-D transgene, and the transfected cells as administered to the mammalian subject. Exemplary procedures for seeding a vascular graft with genetically modified endothelial cells are described in U.S. Patent No. 5,785,965, incorporated herein by reference.

If the mammalian subject is receiving a vascular graft, the VEGF-C or VEGF-D therapeutic composition may be directly applied to the isolated vessel segment prior to its being grafted *in vivo*.

In another preferred embodiment, the administering comprises implanting an intravascular stent in the mammalian subject, where the stent is coated or impregnated with the therapeutic VEGF-C/D gene/protein composition. Exemplary materials for constructing a drug-coated or drug-impregnated stent are described in literature cited above and reviewed in Lincoff *et al., Circulation, 90*: 2070-2084 (1994). As shown in Figure 4A and 4B, a metal or polymeric wire 70 for forming a stent is coated with a composition 72 such as a porous biocompatible polymer or gel that is impregnated with (or can be dipped in or otherwise easily coated immediately prior to use with) a VEGF-C or VEGF-D therapeutic composition. The wire is coiled, woven, or otherwise formed into a stent 74 suitable for implanation into the lumen of a vessel using conventional materials and techniques, such as intravascular angioplasty catheterization. Exemplary stents that may be improved in this manner are described and depicted in U.S. Patent Nos. 5,800,507 and 5,697,967 (Medtronic, Inc., describing an intraluminal stent comprising fibrin and an elutable drug capable of providing a treatment of restenosis); U.S. Patent No. 5,776,184 (Medtronic, Inc., describing a stent with a porous coating comprising a polymer and a therapeutic substance in a solid or solid/solution with the polymer); U.S. Patent No. 5,799,384 (Medtronic, Inc., describing a flexible, cylindrical, metal stent having a biocompatible polymeric surface to contact a body lumen); U.S. Patent Nos. 5,824,048 and 5,679,400; and U.S. Patent No. 5,779,729; all of which are specifically incorporated herein by reference in the entirety. Implantation of such stents during conventional

angioplasty techniques will result in less restenosis than implantation of conventional stents. In this sense, the biocompatibility of the stent is improved.

In another preferred embodiment, the composition comprises microparticles composed of biodegradable polymers such as PGLA, non-degradable polymers, or biological polymers (e.g., starch) which particles encapsulate or are impregnated by the VEGF-C or VEGF-C polypeptide/polynucleotide. Such particles are delivered to the intravascular wall using, e.g., an infusion angioplasty catheter. Other techniques for achieving locally sustained drug delivery are reviewed in Wilensky *et al., Trends Caridovasc. Med., 3:*163-170 (1993), incorporated herein by reference.

Administration via one or more intravenous injections subsequent to the angioplasty or bypass procedure also is contemplated. Localization of the VEGF-C or VEGF-D polypeptides to the site of the procedure occurs due to expression of VEGF-C/D receptors on proliferating endothelial cells. Localization is further facilitated by recombinantly expressing the VEGF-C or VEGF-D as a fusion polypeptide (*e.g.*, fused to an apolipoprotein B-100 oligopeptide as described in Shih *et al., Proc. Nat'l. Acad. Sci. USA, 87:*1436-1440 (1990).

The pharmaceutical efficacy of VEGF-C polynucleotides, VEGF-C polypeptides, VEGF-D polynucleotides, and VEGF-D polypeptides to prevent stenosis or restenosis of a blood vessel is demonstrated *in vivo, e.g.*, using procedures such as those described in the following examples, some of which are prophetic. The examples assist in further describing the invention, but are not intended in any way to limit the scope of the invention.

Example 1

Use of adenovirus-mediated VEGF-C gene transfer to prevent restenosis

The following experiments, performed *in vivo* in a rabbit restenosis model, demonstrate the efficacy of adenovirus-mediated intravascular VEGF-C gene transfer for the prevention of post-angioplasty restenosis.

A. Materials and Methods

1. Adenoviral constructs.

An adenovirus plasmid containing a cDNA encoding the complete human prepro-VEGF-C open reading frame operably linked to a cytomegalovirus (CMV) promoter and human growth hormone polyadenylation signal sequence was constructed as follows. A DNA fragment comprising a CMV promoter sequence was prepared by digesting the pcDNA3.1+ vector (Invitrogen) with *Sal* I and filling-in the 5' overhangs with the Klenow enzyme. The CMV promoter (nucleotides 5431-911) was excised from the vector with *Hind* III and isolated. A full-length human VEGF-C cDNA containing the 1997 bp sequence specified in SEQ ID NO: 1 (as well as less than 50 bases of additional non-coding and polylinker sequence) was excised from a VEGF-C pREP7 expression vector [described in WO 98/33917] with *Hind* III and *Xho* I and isolated. A human growth hormone polyadenylation signal (~ 860 bp) was excised from an αMHC vector with *Sal*I and *Bam*HI. The CMV promoter, VEGF-C cDNA, and hGH polyadenylation signal fragments were simultaneously ligated into a *Bam*HI and *Eco*RV-digested pCRII vector. The ligated CMV promoter and VEGF-C cDNA is shown in SEQ ID NO: 17. The resulting construct was opened with *Bgl*II and partially-digested with *Bam*HI. The full transcriptional unit was ligated into *Bgl*II-opened pAdBglII vector. This construct [designated pAdBg1II VEGF-C] was then used to create recombinant adenovirus containing the CMV-VEGF-C-hGH transcriptional unit, using standard homologous recombination techniques. [Barr *et al.*, *Gene Ther.*, *1*: 51-58 (1994).] Replication-deficient E1-E3 deleted adenoviruses were produced in 293 cells and concentrated by ultracentrifugation using techniques known in the literature. [See, *e.g.*, Barr *et al.* (1994).] A control plasmid comprising the *lacZ* gene operably linked to the same promoter was also used. [Laitinen M. *et al.*, *Hum. Gene Ther.*, *9*: 1481-1486 (1998).] The *lacZ* adenovirus had a nuclear targeted signal, to direct the β-galactosidase expression to the nucleus. Replication-deficient E1-E3 deleted adenoviruses were produced in 293 cells and concentrated by ultracentrifugation (Barr *et al.*, 1994). The adenoviral preparations were analyzed for the absence of helper viruses and bacteriological contaminants.

2. Animal model.

New Zealand White rabbits were employed for the gene transfer study. A first group of rabbits was fed a 0.25 % cholesterol diet for two weeks, then subjected to balloon denudation of the aorta, then subjected three days later to the adenovirus-mediated gene transfer. A second group of rabbits was only subjected to the gene transfer. Animals were sacrificed 2 or 4 weeks after the gene transfer. The number of experimental (VEGF-C) and control (*lacZ*) animals in both study groups was 6.

In the first group of rabbits, the whole aorta, beginning from the tip of the arch, was denuded using a 4.0 F arterial embolectomy catheter (Sorin Biomedical, Irvine, CA). The catheter was introduced via the right iliac artery up to the aortic arch and inflated, and the aorta was denuded twice.

3. Gene transfer.

The gene transfer was performed using a 3.0 F channel balloon local drug delivery catheter (Boston Scientific Corp., Maple Grove, MA). Using fluoroscopical control, the balloon catheter was positioned caudal to the left renal artery, in a segment free of side branches, via a 5 F percutaneous introducer sheath (Arrow International, Reading, PA) in the right carotid artery and inflated to 6 ATM with a mixture of contrast media and saline. The anatomical location of the balloon catheter was determined by measuring its distance from the aortic orifice of the left renal artery. Virus titer of 1.15 x 10^{10} plaque forming units (pfu) was administered to each animal in a final volume of 2 ml (0.9 % NaCl), and the gene transfer was performed at 6 ATM pressure for 10 minutes (0.2 ml / min). In the second study group the animals had only gene transfer and they were sacrificed 2 weeks after the gene transfer. The number of animals in each study group (0.9 % NaCl only; *lacZ* gene transfer; and VEGF-C gene transfer) was 3. All studies were approved by Experimental Animal Committee of the University of Kuopio in Finland.

4. Histology.

Three hours before sacrifice, the animals were injected intravenously with 50 mg of BrdU dissolved in 40 % ethanol. After the sacrifice, the aortic segment where the gene transfer had been performed was removed, flushed gently with saline, and divided into five equal segments. The proximal segment was snap frozen in liquid nitrogen and stored at -70°C. The next segment was immersion-fixed in 4 % paraformaldehyde / 15 %

sucrose (pH 7.4) for 4 hours, rinsed in 15 % sucrose (pH 7.4) overnight, and embedded in paraffin. The medial segment was immersion-fixed in 4 % paraformaldehyde / phosphate buffered saline (PBS) (pH 7.4) for 10 minutes, rinsed 2 hours in PBS, embedded in OCT compound (Miles), and stored at -70°C. The fourth segment was immersion-fixed in 70 % ethanol overnight and embedded in paraffin. The distal segment was directly stained for β-galactosidase activity in X-GAL staining solution at +37°C for 16 hours, immersion-fixed in 4 % paraformaldehyde / 15 % sucrose (pH 7.4) for 4 hours, rinsed in 15 % sucrose overnight, and embedded in paraffin. Paraffin sections were used for immunocytochemical detection of smooth muscle cells (SMC), macrophages, and endothelium. Gene transfer efficiency was evaluated using X-GAL staining of OCT-embedded tissues. BrdU-positive cells were detected according to manufacturer's instructions. Morphometry was performed using haematoxylin-eosin stained paraffin sections using image analysis software. Measurements were taken independently by two observers from multiple sections, without knowledge of the origin of the sections. Intima/media (I/M) ratio was used as a parameter for intimal thickening.

B. Results.

Histological analysis of the balloon-denuded mice revealed that the *lacZ*-transfected control group had an I/M ratio of 0.61 two weeks after the gene transfer, which represented a statistically significant difference ($p < 0.05$) from the VEGF-C-transfected groups (I/M ratio of 0.40). The tendency that VEGF-C group had a smaller I/M ratio persisted at 4 weeks time point after the gene transfer.

In the second group of rabbits that were subjected only to gene transfer to the vessel wall (without endothelial denudation), the I / M ratio in the *lacZ* group was 0.3, compared to 0.15 for the VEGF-C group. This difference, too, represented a statistically significant ($p < 0.05$) inhibition in neointima formation in VEGF-C group.

The BrdU labeling will permit analysis of smooth muscle cell proliferation in VEGF-C-transfected versus control (*lacZ*) animals. SMC proliferation is expected to be reduced in the VEGF-C-transfected population.

The foregoing data demonstrate that VEGF-C gene transfer significantly reduced intimal thickening at two weeks time point after aortic denudation and after vessel wall damage caused by the gene transfer catheter without balloon denudation. These data

indicate a therapeutic utility for VEGF-C gene transfer for the prevention of post-angioplasty restenosis.

Example 2

Comparative Example Demonstrating that Anti-Restenosis Effects of VEFG-C Appear Superior to Those of VEGF.

The following experiments demonstrate the efficacy of adenovirus-mediated intravascular VEGF and VEGF-C gene transfer for the prevention of post-angioplasty restenosis, and demonstrates that VEGF-C appeared to provide a superior therapeutic efficacy compared to VEGF.

A. Materials and Methods

1. Adenoviral constructs.

VEGF (murine VEGF-A_{164}; SEQ ID NO: 18) adenovirus was constructed using the same promoter as the VEGF-C construct, and following similar procedure as described in Example 1. The VEGF-A_{164} adenoviral construct was produced in 293T cells and concentrated essentially as described in Example 1, and analyzed to be free of helper virus, lipopolysaccharides, and bacterial contaminants.

2. Animal model.

Sixty three New Zealand White rabbits were divided into two major groups, the first having 0.25% cholesterol diet for two weeks and balloon denudation of the aorta before gene transfer, and the second group having only the gene transfer. Gene transfer was performed in the first group of rabbits three days after denudation, and the animals were sacrificed 2 or 4 weeks after the gene transfer. Number of rabbits in each study group (*lacZ*, VEGF, and VEGF-C) at both time points was 6. In the second study group, the rabbits had only the gene transfer, without cholesterol diet or balloon denudation, and were sacrificed 2 or 4 weeks after the gene transfer. The number of rabbits in each study group (0.9% saline, *lacZ*, VEGF, and VEGF-C) was 3.

3. Gene transfer.

Gene transfer was performed according to the procedure described in Example 1.

4. Histology

Histology was performed essentially as described in Example 1 with the following modifications: SMC were detected using HHF35 (DAKO, 1:50 dilution), macrophages were detected using RAM-11 (DAKO, 1:50 dilution), endothelium was detected using CD31 (DAKO, 1:50 dilution), and T cells were detected using MCA 805 (DAKO, 1:100 dilution). Controls for immunostainings included incubations with class- and species matched immunoglobulins and incubations where primary antibodies were omitted. Morphometry and image analysis were performed using Image-Pro Plus™ software and an Olympus AX70 microscope (Olympus Optical, Japan). Statistical analyses were performed using the ANOVA and modified *t*-test. $P<0.05$ was considered statistically significant.

B. Results

Histological analysis of the balloon-denuded rabbit aorta shows intimal thickening and SMC proliferation. Two weeks after gene transfer, the *lacZ* control group had the highest I/M ratio (0.57 ± 0.04) whereas VEGF-C (0.38 ± 0.02) and VEGF (0.49 ± 0.17) groups showed decreased intimal thickening. The difference in I/M ratios between *lacZ* and VEGF-C groups was significant ($P<0.05$), whereas those between *lacZ* and VEGF groups were not statistically significant, at the two-week time point. The tendency that both VEGF and VEGF-C groups had smaller I/M ratios persisted at the four week time point when the I/M ratio was 0.73 ± 0.16, 0.44 ± 0.14, and 0.63 ± 0.21 for the lacZ, VEGF-C, and VEGF groups, respectively. Hematoxylin-eosin and immunostainings of the transfected arteries indicate that intimal thickening in all arteries was composed predominantly of SMC.

Use of adenoviral vectors can lead to immnological and inflammatory responses, partly because high titer adenovirus induces expression of NFκB and activates a CTL response. However, no signs of inflammation nor foam cell accumulation were detected as judged by macrophage and T-cell immunostainings. In addition, human clinical gene therapy grade viruses were used together with short exposure times in the transfected arteries, which may also help explain the absence of severe inflammatory reactions in this study.

The percentage of proliferating cells was analyzed using BrdU labeling. No

significant differences were seen, although the VEGF-C group tended to have a lower proliferation rate, consistent with the observation that VEGF-C transduced arteries had smaller I/M ratios at both time points. Two weeks after balloon denudation, the percentage of proliferating cells was 1.8 ± 0.4, 2.2 ± 0.7, and 1.2 ± 0.0 for the *lacZ*, VEGF, and VEGF-C groups, respectively, and after four weeks, the percentage of proliferating cells was 0.3 ± 0.1, 1.2 ± 0.5, and 0.3 ± 0.1 for the *lacZ*, VEGF, and VEGF-C groups, respectively. Endothelial regrowth was analyzed by measuring the length of intact endothelium from histological sections. No significant differences were found between the study groups.

The potential of adenovirus to cause damage to the vessel wall and neointima formation was tested by performing high-titer adenovirus gene transfer to intact abdominal aorta of rabbits without balloon-denudation. Control rabbits were treated in the same way with 0.9% saline. The positioning of the gene transfer catheter caused some internal elastic lamina damage and moderate induction of neoinitma formation after the procedure. At the two-week time point, the I/M ratio in the *lacZ* group was 0.24 ± 0.06, in the control group 0.28 ± 0.05, in the VEGF-C group 0.18 ± 0.07, and in the VEGF group 0.15 ± 0.03. At the four-week time point the *lacZ* group had an I/M ratio of 0.22 ± 0.13, the VEGF-C group 0.13 ± 0.03, and the VEGF group 0.23 ± 0.11.

This study shows a beneficial therapeutic effect of intravascular adenovirus-mediated VEGF-C gene transfer on the vessel wall after balloon injury, and also compares VEGF-C and VEGF adenovirus-mediated gene transfer for the prevention of neointima formation. Although different receptor binding profiles of VEGF-C and VEGF might have led to different biological effects in the vessel wall, both VEGFs reduced intimal thickening two weeks after gene transfer. Thus, both VEGFs are potential candidates for vascular gene therapy of ischemic atherosclerotic diseases. However, according to this experiment, VEGF-C appears to prevent restenosis more effectively than VEGF in this model system. The superior ability of VEGF-C to prevent restenosis, as compared to VEGF, could be due to expression or activity of VEGFR-3 which is a receptor for VEGF-C and VEGF-D, but not for VEGF. Alternatively, the apparent superiority may be attributable to a restenosis-promoting effect of VEGF mediated through VEGFR-1 or due to differential ligand effects (VEGF-C versus VEGF) mediated thru the common receptor VEGFR-2,

which is reportedly expressed in vascular smooth muscle cells. [See Grosskreutz *et al.*, *Microvasc. Res., 58(2)*: 128-136 (September, 1999).

Example 3

Expression of transfected VEGFs in the aortic wall

Using the aortic segments from the same experimental animals described in Example 2, mRNA expression of *lacZ*, VEGF-C and VEGF (murine VEGF-A_{164}) was analyzed in aortic tissue after gene transfer. Total RNA was extracted from transfected aortic segments using Trizol Reagent (Gibco-BRL), and 2 μg of RNA was used for cDNA synthesis. Primers for *lacZ*, VEGF-C and VEGF were designed to distinguish between endogenous and transduced genes by selecting the 5' primers from the CMV promoter and the 3' primers from the coding regions.

For *lacZ* amplification, primers were: 5' primer 5'-TTGGAGGCCTAGGCTTTTGC-3' (SEQ ID NO: 5) and 3' primer 5'-ATACTGTCGTCGTCCCCTCA-3' (SEQ ID NO: 6). The first PCR cycle was an initial incubation at 96°C for 4 minutes followed by 80°C for 3 minutes during which the DNA polymerase was added. This was followed by 30 cycles, each consisting of 94°C for 45 seconds, 58°C for 45 seconds, and 72°C for 50 seconds, followed by a final extension of 72°C for 5 minutes. 5 μl of the first PCR product was used for the second PCR with 5' primer 5'-GGTAGAAGACCCCAAGGACTTT-3'(SEQ ID NO: 7) and 3' primer 5'-CGCCATTCGCCATTCAG-3' (SEQ ID NO: 8). The first PCR cycle was an initial incubation at 96°C for 3 minutes followed by 80°C for 3 minutes followed by 32 cycles, each consisting of 94°C for 60 seconds, 58°C for 15 seconds, and 72°C for 90 seconds, followed by a final extension of 72°C for 5 minutes.

For VEGF-C amplification, primers were: 5' primer 5'-CTGCTTACTGGCTTATCG-3' (SEQ ID NO: 9) and 3' primer 5'-CCTGTTCTCTGTTATGTTGC-3' (SEQ ID NO: 10). The first PCR cycle was an initial incubation at 96°C for 4 minutes followed by 80°C for 3 minutes during which the DNA polymerase was added. This was followed by 39 cycles each consisting of 94°C for 30 seconds, 56°C for 40 seconds, and 72°C for 90 seconds, followed by a final extension of 72°C for 5 minutes. 5 μl of the first PCR product was used for the second PCR with 5'

primer 5'-TCTCCAAAAAGCTACACCG-3' (SEQ ID NO: 11) and 3' primer 5'-CAAGTGCATGGTGGAAGG-3' (SEQ ID NO: 12). The first PCR cycle was an initial incubation at 96°C for 3 minutes followed by 80°C for 3 minutes followed by 39 cycles each consisting of 94°C for 60 seconds, 57°C for 30 seconds, and 72°C for 90 seconds, followed by a final extension of 72°C for 5 minutes.

For VEGF amplification, primers were: 5' primer 5'-TCGATCCATGAACTTTCTGC-3' (SEQ ID NO: 13) and 3' primer 5'-TTCGTTTAACTCAAGCTGCC-3' (SEQ ID NO: 14). The first PCR cycle was an initial incubation at 96°C for 4 minutes followed by 80°C for 3 minutes, followed by 39 cycles each consisting of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 90 seconds, followed by a final extension of 72°C for 5 minutes. 5 µl of the first PCR product was used for the second PCR with 5' primer 5'-GACCCTGGCTTTACTGCTG-3' (SEQ ID NO: 15) and 3' primer 5'-GGAACATTTACACGTCTGCG-3' (SEQ ID NO: 16). The first PCR cycle was an initial incubation at 96°C for 3 minutes followed by 80°C for 3 minutes followed by 39 cycles each consisting of 94°C for 60 seconds, 54°C for 30 seconds, and 72°C for 90 seconds, followed by a final extension of 72°C for 5 minutes.

The mRNA of *lacZ*, VEGF-C and VEGF was detected in aortic wall tissue up to four weeks after gene transfer.

Gene transfer efficiency was evaluated by assaying *lacZ* expression, analyzed by X-Gal staining for β-galactosidase activity, in OCT embedded tissue sections. Transfection efficiency was 1.1% ± 0.5 and 0.3% ± 0.1, two and four weeks respectively, after intravascular catheter-mediated gene transfer.

Example 4

Expression of VEGF receptors in the aortic wall

Using the experimental animals described in Example 2, VEGFR-1, VEGFR-2, and VEGFR-3 expression in aortic tissue was analyzed by immunostainings and *in situ* hybridization. Immunohisochemistry was performed using clone sc-316 (Santa Cruz Biotechnology, 1:50 dilution) to detect VEGFR-1, clone sc-6251 (Santa Cruz Biotechnology, 1:500 dilution) to detect VEGFR-2, and clone sc-637 (Santa Cruz Biotechnology, 1:300 dilution) to detect VEGFR-3. Controls for immunostainings

included incubations with class- and species matched immunoglobulins and incubations where primary antibodies were omitted. *In situ* hybridization of VEGF receptor mRNAs was carried out using ^{33}P-UTP labeled riboprobes. Expression of all receptors was localized to endothelium. VEGFR-2 was also expressed in neointimal SMCs.

Example 5

Use of naked VEGF-C transgene therapy to prevent restenosis

The procedures described in Example 1 or 2 are repeated, with the following modifications. Instead of using an adenovirus vector for delivery of the VEGF-C transgene, a mammalian expression vector is constructed for direct gene transfer (of naked plasmid DNA). The VEGF-C coding sequence is operably linked to a suitable promoter, such as the CMV promoter, and preferably linked to a suitable polyadenylation sequence, such as the human growth hormone polyadenylation sequence. Exemplary VEGF-C vectors can be modeled from vectors that have been described in the literature to perform vector-free gene transfer for other growth factors, by substituting a VEGF-C coding sequence for a VEGF coding sequence. [See, *e.g.*, Isner *et al.*, *Circulation, 91*: 2687-2692 (1995); and Isner *et al.*, *Human Gene Therapy, 7*: 989-1011 (1996), incorporated herein by reference.] vector. A similar construct comprising a *lacZ* gene is used as a control.

A Hydrogel-coated balloon catheter (Boston Scientific) is used to deliver the VEGF-C transgene essentially as described in Asahara *et al.*, *Circulation, 94*: 3291-3302 (December 15, 1996), incorporated herein by reference. Briefly, an angioplasty balloon is prepared *ex vivo* by advancing the deflated balloon completely through a teflon protective sheath (Boston Scientific). The balloon is inflated and a conventional pipette is used to apply the transgene construct (*e.g.*, 50 - 5000 µg transgene DNA in a saline solution) to the Hydrogel polymer coating the external surface of the inflated balloon. After the transgene solution has dried, the balloon is deflated, withdrawn into the protective sheath, and re-inflated to minimize blood flow across the balloon surface until the balloon is properly positioned in the target artery.

Intima/media (I/M) ratio is again used as a parameter for intimal thickening. Reduced I/M ratio in animals treated with the VEGF-C transgene-coated balloon catheter

is considered indicative of therapeutic efficacy. As described in Example 2, comparison of the therapeutic efficacy of VEGF-C gene transfer with other therapies, such as VEGF gene transfer, can be conducted in parallel.

Example 6

Use of VEGF-C gene therapy to prevent restenosis following angioplasty with stent

The procedures described in the preceding examples are repeated with the modification that initial balloon angioplasty is accompanied by implantation of a coronary stent using conventional procedures. The VEGF-C transgene is delivered concurrently or immediately before or after stent implantation essentially as described in the preceding examples. Increased quantities (*e.g.*, two-fold to ten-fold) of the transgene (compared to angioplasty without stent) and increased transfection time may be desirable, as described in Van Belle *et al., J. Am. Coll. Cardiol., 29*:1371-1379 (May, 1997), incorporated by reference herein. Decreased neointimal thickening and/or decreased thrombotic occlusion in the VEGF-C gene-treated animals versus control animals treated with a marker gene is considered evidence of the efficacy of the VEGF-C gene therapy.

Example 7

Use of an extravascular collar to reduce vascular stenosis.

An inert silicone collar such as described in International Patent Publication No. WO 98/20027 is surgically implanted around the carotid arteries of New Zealand White Rabbits. The collar acts as an irritation agent that will induce intimal thickening, and contains a reservoir suitable for local delivery of a VEGF-C transgene or protein pharmaceutical formulation. Gene transfer, using the VEGF-C adenovirus construct or control construct described in Example 1 is initiated five days later by injecting 10^8-10^{11} pfu into the collar. Animals are sacrificed 14 or 28 days later and histological examinations are performed as described in Example 1. Intima/media thickness ratio [Yla-Herttuala *et al., Arteriosclerosis, 6*: 230-236 (1986)] is used as an indicia of stenosis. Reduced I/M ratio in the VEGF-C-transfected rabbits, as compared to the *lacZ* control rabbits, indicates therapeutic efficacy of VEGF-C gene transfer for preventing arterial stenosis.

Example 8

Use of VEGF-C polypeptides to reduce or prevent restenosis

The procedures described in Example 1 are repeated except, instead of treating the test animals with an adenovirus containing a VEGF-C transgene or *lacZ* control, the animals are treated with a composition comprising a VEGF-C polypeptide in a pharmaceutically acceptable carrier (*e.g.*, isotonic saline with serum albumim), or with carrier solution alone as a control. Test animals receive either 10, 100, 250, 500, 1000, or 5000 μg of a VEGF-C polypeptide via intra-arterial infusion, *e.g.*, as described in Example 1. A second group of animals additionally receive an injection of the VEGF-C polypeptide 7 days later. The animals are sacrificed and histological examination performed as described in Example 1. Reduced I/M ratio in the VEGF-C-treated animals versus control animals provides evidence of the therapeutic efficacy of VEGF-C polypeptide treatment. Repetition of the experiment using various sustained-release VEGF-C formulations and materials as described above is expected to further enhance the therapeutic efficacy of the VEGF-C polypeptide. Moreover, a treatment regimen comprising the simultaneous administration of VEGF-C protein (to provide immediate therapy to the target vessel) with a VEGF-C transgene (to provide sustained therapy for several days or weeks) is specifically contemplated as a variation of the invention.

Example 9

Anti-stenosis/anti-restenosis activity of VEGF-D

The procedures described in the preceding examples are repeated using a composition comprising a VEGF-D polynucleotide or VEGF-D polypeptide in lieu of the VEGF-C polynucleotide/polypeptide, to demonstrate the ability of VEGF-D to prevent stenosis or restenosis of a blood vessel.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those in the art, all of which are intended as aspects of the present invention. Accordingly, only such limitations as appear in the claims should be placed on the invention.

CLAIMS

1. A method of treating a mammalian subject to prevent stenosis or restenosis of a blood vessel, comprising the step of:

administering to a mammalian subject in need of treatment to prevent stenosis or restenosis of a blood vessel a composition comprising at least one anti-restenosis agent selected from the group consisting of a polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor C (VEGF-C) polypeptide, a polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor D (VEGF-D) polypeptide, a VEGF-C polypeptide, and a VEGF-D polypeptide, thereby preventing stenosis or restenosis of said blood vessel.

2. A method according to claim 1 wherein said mammalian subject is human.

3. A method according to claim 1 or 2 wherein said composition comprises a polynucleotide that comprises a nucleotide sequence encoding a mammalian VEGF-C polypeptide.

4. A method according to any of claims 1-3 wherein said composition comprises a polynucleotide that comprises a nucleotide sequence that encodes a human VEGF-C polypeptide.

5. A method according to claim 4 wherein said VEGF-C polypeptide comprises an amino acid sequence comprising a continuous portion of SEQ ID NO: 2, said continuous portion having, as its amino terminus, an amino acid selected from the group consisting of positions 30-131 of SEQ ID NO: 2, and having, as its carboxyl terminus, an amino acid selected from the group consisting of positions 211 to 419 of SEQ ID NO: 2.

6. A method according to claim 5 wherein said polynucleotide further comprises a nucleotide sequence encoding a secretory signal peptide, and wherein the sequence encoding the secretory signal peptide is connected in-frame with the sequence that encodes the VEGF-C polypeptide

7. A method according to claim 6 wherein said polynucleotide lacks a nucleotide sequence encoding amino acids 228-419 of SEQ ID NO: 2.

8. A method according to claim 6 or 7 wherein said polynucleotide lacks a nucleotide sequence encoding amino acids 32-102 of SEQ ID NO: 2.

9. A method according to any one of claims 6-8 wherein the polynucleotide further comprises a promoter sequence operably connected to the sequence that encodes the secretory signal sequence and VEGF-C polypeptide, wherein the promoter sequence promotes transcription of the sequence that encodes the secretory signal sequence and the VEGF-C polypeptide in cells of the mammalian subject.

10. A method according to claim 9 wherein the polynucleotide further comprises a polyadenylation sequence operably connected to the sequence that encodes the VEGF-C polypeptide.

11. A method according to any one of claims 3-10 wherein the composition comprises a gene therapy vector, said gene therapy vector comprising said polynucleotide.

12. A method according to claim 11 wherein said vector comprises a replication-deficient adenovirus, said adenovirus comprising the polynucleotide operably connected to a promoter and flanked by adenoviral polynucleotide sequences.

13. A method according to any one of claims 1-12 wherein the composition further comprises a pharmaceutically acceptable carrier.

14. A method according to any one of claims 1-13 wherein said administering comprises at least one intravascular injection of said composition.

15. A method according to any one of claims 1-13 wherein said administering comprises a catheter-mediated transfer of said composition into a blood vessel of the mammalian subject.

16. A method according to claim 15 wherein said catheter-mediated gene transfer comprises introducing a catheter into a coronary artery of the mammalian subject, and releasing the composition into the coronary artery.

17. A method according to any one of claims 1-16 wherein said administering is conducted in said human concurrently with a percutaneous transluminal coronary angioplasty.

18. A method according to claim 1 wherein the composition comprises a VEGF-C polypeptide, in an amount effective to prevent stenosis or restenosis of said blood vessel.

19. A method according to claim 18 wherein said administering comprises implanting an intravascular stent in said mammalian subject, and wherein the stent is coated or impregnated with the composition.

20. A treatment to prevent stenosis or restenosis of a blood vessel in a human, comprising delivering a replication-deficient adenovirus vector to the vessel, said vector comprising a polynucleotide encoding at least one of polypeptide selected from a VEGF-C polypeptide and a VEGF-D polypeptide, and further comprising a promoter sequence to promote expression of the at least one polypeptide in cells of the blood vessel, thereby preventing stenosis or restenosis of the blood vessel.

21. A method according to claim 20 wherein the polynucleotide encodes a VEGF-C polypeptide.

22. A medical device designed to contact a surface of a blood vessel in the course of surgery to treat stenosis of the blood vessel, the device characterized by an improvement comprising integrating into the device a composition effective to prevent restenosis, said composition comprising at least one anti-restenosis agent selected from the group consisting of a VEGF-C polynucleotide, a VEGF-C polypeptide, a VEGF-D polynucleotide, and a VEGF-D polypeptide.

23. The medical device of claim 22, wherein the device is selected from the group consisting of intravascular stents, intravascular catheters, extravascular collars, elastomeric membranes adapted to cover a surface of an intravascular stent or catheter, and combinations thereof.

24. A medical device according to claim 22 comprising an endovascular stent having an outer surface for contacting a surface of a blood vessel, and a composition on said surface, said composition comprising at least one anti-restenosis agent selected from the group consisting of a VEGF-C polynucleotide, a VEGF-C polypeptide, a VEGF-D polynucleotide, and a VEGF-D polypeptide.

25. A medical device according to claim 22 comprising a catheter having an outer surface for contacting a surface of a blood vessel, and a composition on said surface, said composition comprising at least one member selected from the group consisting of a VEGF-C polynucleotide, a VEGF-C polypeptide, a VEGF-D polynucleotide, and a VEGF-D polypeptide.

26. A medical device according to claim 22 comprising a balloon catheter having a void for holding a therapeutic agent for delivery to the interior of a blood vessel, and a composition contained in the void, the composition comprising at least one anti-restenosis agent selected from the group consisting of a VEGF-C polynucleotide, a VEGF-C polypeptide, a VEGF-D polynucleotide, and a VEGF-D polypeptide.

27. A kit for treating restenosis comprising a container holding at least one anti-restenosis agent selected from the group consisting of a VEGF-C polynucleotide, a VEGF-C polypeptide, a VEGF-D polynucleotide, and a VEGF-D polypeptide; and a label attached to or packaged with the container, the label describing use of the compound for prevention of restenosis of a blood vessel.

28. A kit according to claim 27, further comprising a medical device selected from the group consisting of: intravascular stents, intravascular catheters, extravascular collars, and membranes adapted to cover a surface of an intravascular stent or catheter.

29. The use of a composition for the manufacture of a medicament for the treatment or prevention of stenosis or restenosis of a blood vessel, said composition comprising at least one anti-restenosis agent selected from the group consisting of a polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor C (VEGF-C) polypeptide, a polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor D (VEGF-D) polypeptide, a VEGF-C polypeptide, and a VEGF-D polypeptide.

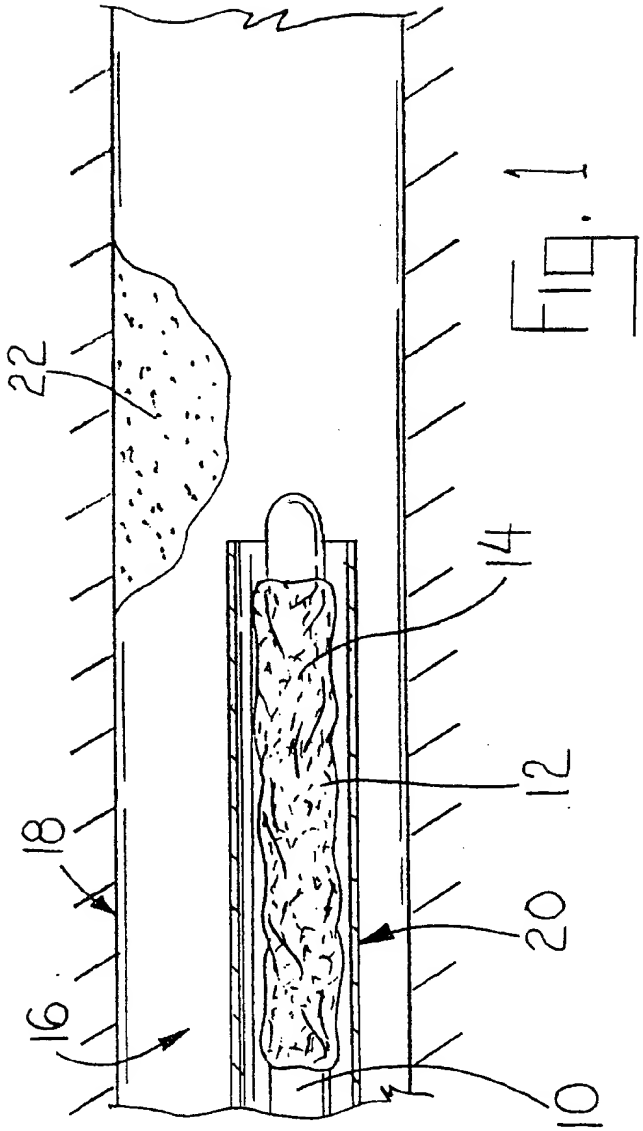

FIG. 1

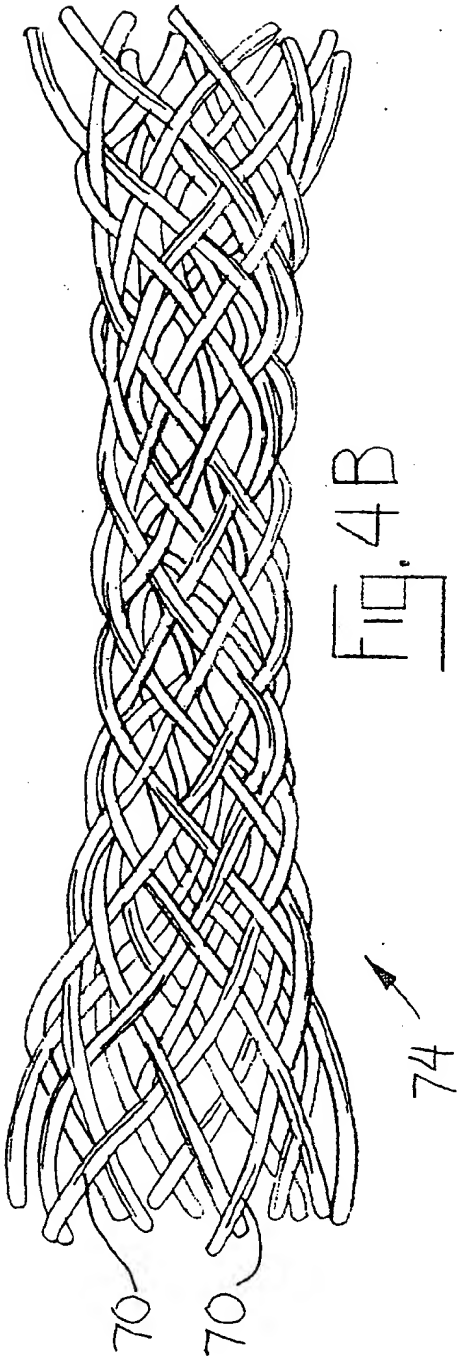

FIG. 4B

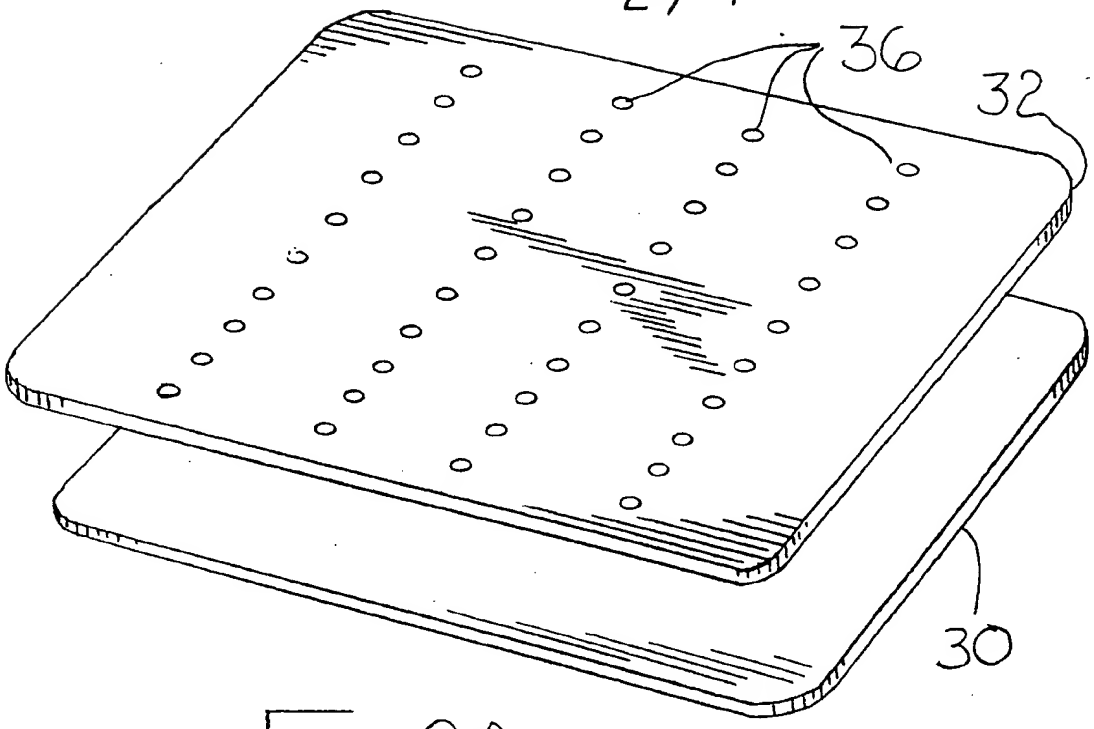

Fig. 2A

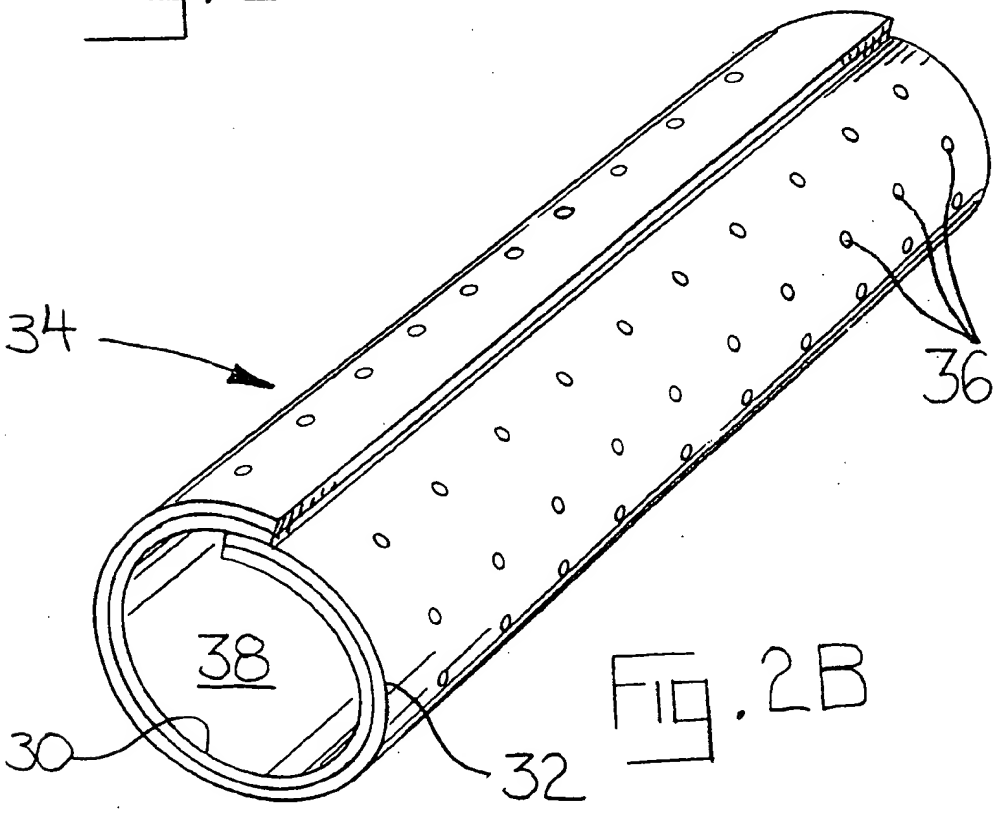

Fig. 2B

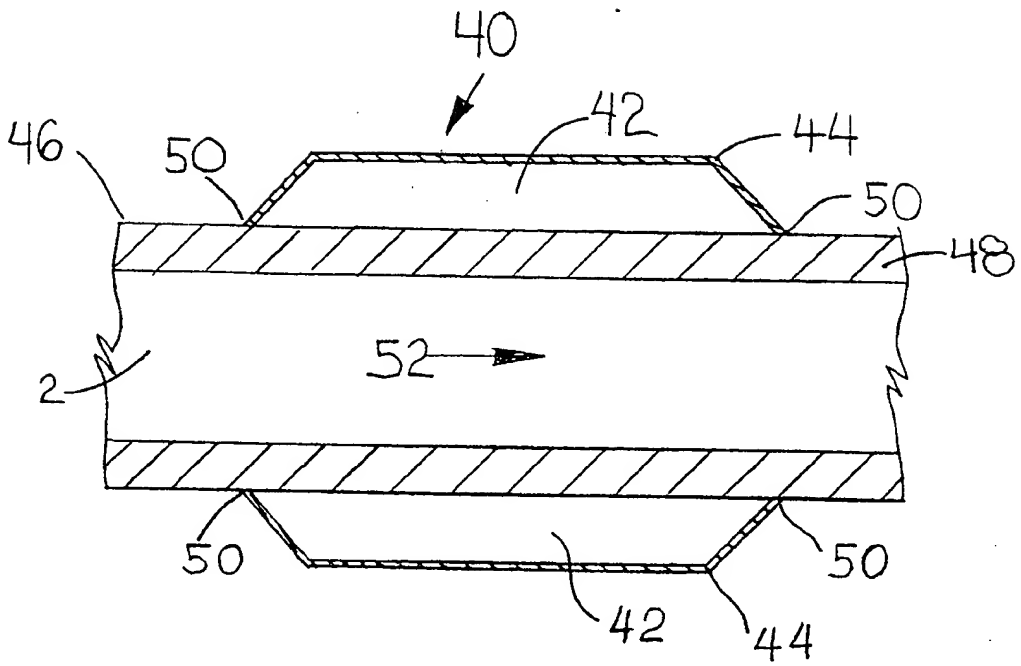

Fig. 3B

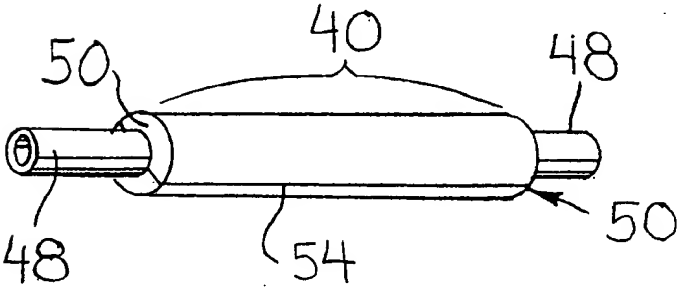

Fig. 3A

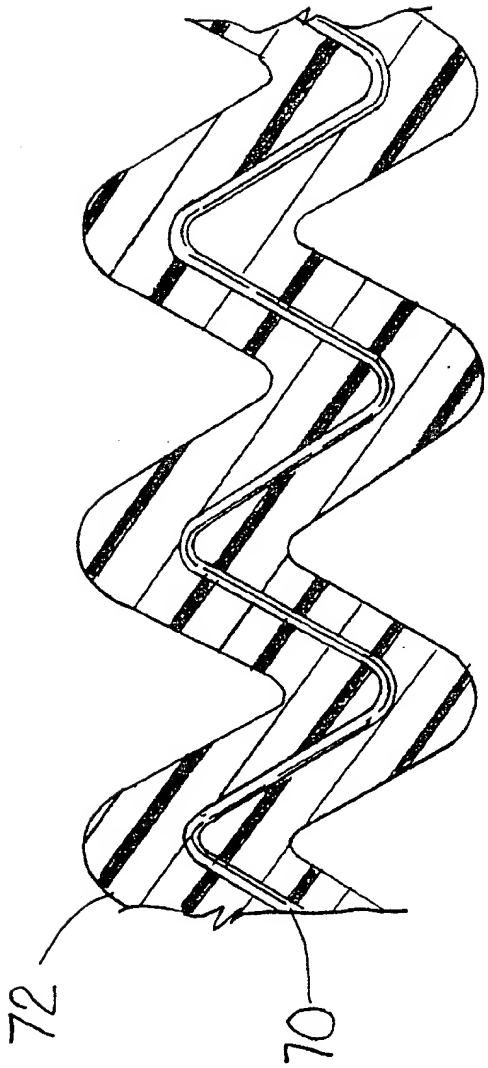

FIG 4A

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Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr
    100                 105                 110

aat aca gag atc ttg aaa agt att gat aat gag tgg aga aag act caa   741
Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln
115                 120                 125                 130

tgc atg cca cgg gag gtg tgt ata gat gtg ggg aag gag ttt gga gtc   789
Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val
                135                 140                 145

gcg aca aac acc ttc ttt aaa cct cca tgt gtg tcc gtc tac aga tgt   837
Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys
            150                 155                 160

ggg ggt tgc tgc aat agt gag ggg ctg cag tgc atg aac acc agc acg   885
Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr
        165                 170                 175

agc tac ctc agc aag acg tta ttt gaa att aca gtg cct ctc tct caa   933
Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln
    180                 185                 190

ggc ccc aaa cca gta aca atc agt ttt gcc aat cac act tcc tgc cga   981
Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg
195                 200                 205                 210

tgc atg tct aaa ctg gat gtt tac aga caa gtt cat tcc att att aga   1029
Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg
                215                 220                 225

cgt tcc ctg cca gca aca cta cca cag tgt cag gca gcg aac aag acc   1077
Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr
            230                 235                 240

tgc ccc acc aat tac atg tgg aat aat cac atc tgc aga tgc ctg gct   1125
Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala
        245                 250                 255

cag gaa gat ttt atg ttt tcc tcg gat gct gga gat gac tca aca gat   1173
Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp
    260                 265                 270

gga ttc cat gac atc tgt gga cca aac aag gag ctg gat gaa gag acc   1221
Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr
275                 280                 285                 290

tgt cag tgt gtc tgc aga gcg ggg ctt cgg cct gcc agc tgt gga ccc   1269
Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro
                295                 300                 305
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cac aaa gaa cta gac aga aac tca tgc cag tgt gtc tgt aaa aac aaa   1317
His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys
            310                 315                 320

ctc ttc ccc agc caa tgt ggg gcc aac cga gaa ttt gat gaa aac aca   1365
Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr
        325                 330                 335

tgc cag tgt gta tgt aaa aga acc tgc ccc aga aat caa ccc cta aat   1413
Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn
    340                 345                 350

cct gga aaa tgt gcc tgt gaa tgt aca gaa agt cca cag aaa tgc ttg   1461
Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu
355                 360                 365                 370

tta aaa gga aag aag ttc cac cac caa aca tgc agc tgt tac aga cgg   1509
Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg
                375                 380                 385

cca tgt acg aac cgc cag aag gct tgt gag cca gga ttt tca tat agt   1557
Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser Tyr Ser
            390                 395                 400

gaa gaa gtg tgt cgt tgt gtc cct tca tat tgg aaa aga cca caa atg   1605
Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro Gln Met
        405                 410                 415

agc taagattgta ctgttttcca gttcatcgat tttctattat ggaaaactgt       1658
Ser

gttgccacag tagaactgtc tgtgaacaga gagacccttg tgggtccatg ctaacaaaga 1718

caaaagtctg tctttcctga accatgtgga taactttaca gaaatggact ggagctcatc 1778

tgcaaaaggc ctcttgtaaa gactggtttt ctgccaatga ccaaacagcc aagattttcc 1838

tcttgtgatt tctttaaaag aatgactata taatttattt ccactaaaaa tattgtttct 1898

gcattcattt ttatagcaac aacaattggt aaaactcact gtgatcaata ttttttatatc 1958

atgcaaaata tgtttaaaat aaaatgaaaa ttgtattat                        1997
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<210> 2
<211> 419
<212> PRT
<213> Homo sapiens

<400> 2

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Met His Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala
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Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Ala Phe
            20                  25                  30

Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala
        35                  40                  45

Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser
    50                  55                  60
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Ser Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met
65 70 75 80

Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln
85 90 95

Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala
100 105 110

His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys
115 120 125

Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe
130 135 140

Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr
145 150 155 160

Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr
165 170 175

Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu
180 185 190

Ser Gln Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser
195 200 205

Cys Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile
210 215 220

Ile Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn
225 230 235 240

Lys Thr Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys
245 250 255

Leu Ala Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser
260 265 270

Thr Asp Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu
275 280 285

Glu Thr Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys
290 295 300

Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys
305 310 315 320

Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu
325 330 335

Asn Thr Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro
340 345 350

Leu Asn Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys
355 360 365

Cys Leu Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr
370 375 380

Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser
385 390 395 400

Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro
405 410 415

Gln Met Ser

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<213> Homo sapiens

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cattttgatt tttttcatct ctctctcccc acccctaaga ttgtgcaaaa aaagcgtacc 240

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tgaagttttg aggtttcaaa ctttccttct ggagaatgcc ttttgaaaca attttctcta 360

gctgcctgat gtcaactgct tagtaatcag tggatattga aatattcaaa atg tac 416
Met Tyr
1

aga gag tgg gta gtg gtg aat gtt ttc atg atg ttg tac gtc cag ctg 464
Arg Glu Trp Val Val Val Asn Val Phe Met Met Leu Tyr Val Gln Leu
5 10 15

gtg cag ggc tcc agt aat gaa cat gga cca gtg aag cga tca tct cag 512
Val Gln Gly Ser Ser Asn Glu His Gly Pro Val Lys Arg Ser Ser Gln
20 25 30

tcc aca ttg gaa cga tct gaa cag cag atc agg gct gct tct agt ttg 560
Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser Ser Leu
35 40 45 50

gag gaa cta ctt cga att act cac tct gag gac tgg aag ctg tgg aga 608
Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp Trp Lys Leu Trp Arg
55 60 65

tgc agg ctg agg ctc aaa agt ttt acc agt atg gac tct cgc tca gca 656
Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met Asp Ser Arg Ser Ala
70 75 80

tcc cat cgg tcc act agg ttt gcg gca act ttc tat gac att gaa aca 704
Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Ile Glu Thr
85 90 95

cta aaa gtt ata gat gaa gaa tgg caa aga act cag tgc agc cct aga 752
Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser Pro Arg
100 105 110

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gaa acg tgc gtg gag gtg gcc agt gag ctg ggg aag agt acc aac aca   800
Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Ser Thr Asn Thr
115                 120                 125                 130

ttc ttc aag ccc cct tgt gtg aac gtg ttc cga tgt ggt ggc tgt tgc   848
Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly Cys Cys
                135                 140                 145

aat gaa gag agc ctt atc tgt atg aac acc agc acc tcg tac att tcc   896
Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser Thr Ser Tyr Ile Ser
            150                 155                 160

aaa cag ctc ttt gag ata tca gtg cct ttg aca tca gta cct gaa tta   944
Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro Glu Leu
        165                 170                 175

gtg cct gtt aaa gtt gcc aat cat aca ggt tgt aag tgc ttg cca aca   992
Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys Cys Leu Pro Thr
    180                 185                 190

gcc ccc cgc cat cca tac tca att atc aga aga tcc atc cag atc cct  1040
Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln Ile Pro
195                 200                 205                 210

gaa gaa gat cgc tgt tcc cat tcc aag aaa ctc tgt cct att gac atg  1088
Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys Pro Ile Asp Met
                215                 220                 225

cta tgg gat agc aac aaa tgt aaa tgt gtt ttg cag gag gaa aat cca  1136
Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln Glu Glu Asn Pro
            230                 235                 240

ctt gct gga aca gaa gac cac tct cat ctc cag gaa cca gct ctc tgt  1184
Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu Pro Ala Leu Cys
        245                 250                 255

ggg cca cac atg atg ttt gac gaa gat cgt tgc gag tgt gtc tgt aaa  1232
Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu Cys Val Cys Lys
    260                 265                 270

aca cca tgt ccc aaa gat cta atc cag cac ccc aaa aac tgc agt tgc  1280
Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys Ser Cys
275                 280                 285                 290

ttt gag tgc aaa gaa agt ctg gag acc tgc tgc cag aag cac aag cta  1328
Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His Lys Leu
                295                 300                 305

ttt cac cca gac acc tgc agc tgt gag gac aga tgc ccc ttt cat acc  1376
Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe His Thr
            310                 315                 320

aga cca tgt gca agt ggc aaa aca gca tgt gca aag cat tgc cgc ttt  1424
Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys Arg Phe
        325                 330                 335

cca aag gag aaa agg gct gcc cag ggg ccc cac agc cga aag aat cct  1472
Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys Asn Pro
    340                 345                 350
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tga ttcagcgttc caagttcccc atccctgtca tttttaacag catgctgctt 1525

355
tgccaagttg ctgtcactgt ttttttccca ggtgttaaaa aaaaaatcca ttttacacag 1585

caccacagtg aatccagacc aaccttccat tcacaccagc taaggagtcc ctggttcatt 1645

gatggatgtc ttctagctgc agatgcctct gcgcaccaag gaatggagag gaggggaccc 1705

atgtaatcct tttgtttagt tttgtttttg ttttttggtg aatgagaaag gtgtgctggt 1765

catggaatgg caggtgtcat atgactgatt actcagagca gatgaggaaa actgtagtct 1825

ctgagtcctt tgctaatcgc aactcttgtg aattattctg attcttttt atgcagaatt 1885

tgattcgtat gatcagtact gactttctga ttactgtcca gcttatagtc ttccagttta 1945

atgaactacc atctgatgtt tcatatttaa gtgtatttaa agaaaataaa caccattatt 2005

caagccaaaa aaaaaaaaaa aaaa 2029

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<212> PRT
<213> Homo sapiens

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Gln Leu Val Gln Gly Ser Ser Asn Glu His Gly Pro Val Lys Arg Ser
20 25 30

Ser Gln Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser
35 40 45

Ser Leu Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp Trp Lys Leu
50 55 60

Trp Arg Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met Asp Ser Arg
65 70 75 80

Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Ile
85 90 95

Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser
100 105 110

Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Ser Thr
115 120 125

Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly
130 135 140

Cys Cys Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser Thr Ser Tyr
145 150 155 160

Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro
165 170 175

Glu Leu Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys Cys Leu

180 185 190

Pro Thr Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln
195 200 205

Ile Pro Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys Pro Ile
210 215 220

Asp Met Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln Glu Glu
225 230 235 240

Asn Pro Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu Pro Ala
245 250 255

Leu Cys Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu Cys Val
260 265 270

Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys
275 280 285

Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His
290 295 300

Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe
305 310 315 320

His Thr Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys
325 330 335

Arg Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys
340 345 350

Asn Pro

<210> 5
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 5
ttggaggcct aggcttttgc 20

<210> 6
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 6
atactgtcgt cgtcccctca 20

<210> 7
<211> 22

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 7
ggtagaagac cccaaggact tt 22

<210> 8
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 8
cgccattcgc cattcag 17

<210> 9
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 9
ctgcttactg gcttatcg 18

<210> 10
<211> 20
<212> DNA
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<220>
<223> Description of Artificial Sequence: primer

<400> 10
cctgttctct gttatgttgc 20

<210> 11
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 11
tctccaaaaa gctacaccg 19

<210> 12
<211> 18
<212> DNA
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<220>
<223> Description of Artificial Sequence: primer

<400> 12
caagtgcatg gtggaagg 18

<210> 13
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 13
tcgatccatg aactttctgc 20

<210> 14
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<212> DNA
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<220>
<223> Description of Artificial Sequence: primer

<400> 14
ttcgtttaac tcaagctgcc 20

<210> 15
<211> 19
<212> DNA
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<220>
<223> Description of Artificial Sequence: primer

<400> 15
gaccctggct ttactgctg 19

<210> 16
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 16
ggaacattta cacgtctgcg 20

<210> 17
<211> 2679
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: chimeric

sequence in which CMV promoter sequence is ligated
to Homo sapien VEGF-C sequence

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agcccatata tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg 120

cccaacgacc cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata 180

gggactttcc attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta 240

catcaagtgt atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc 300

gcctggcatt atgcccagta catgacctta tgggactttc ctacttggca gtacatctac 360

gtattagtca tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga 420

tagcggtttg actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg 480

ttttggcacc aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg 540

caaatgggcg gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact 600

agagaaccca ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa 660

gctggctagc gtttaaactt aacccgcccc gcctctccaa aaagctacac cgacgcggac 720

cgcggcggcg tcctccctcg ccctcgcttc acctcgcggg ctccgaatgc ggggagctcg 780

gatgtccggt ttcctgtgag gcttttacct gacacccgcc gcctttcccc ggcactggct 840

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ctcgcccagg gggggtcgcc gggaggagcc cgggggagag ggaccaggag gggcccgcgg 960

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tgtgtccgtc tacagatgtg ggggttgctg caatagtgag gggctgcagt gcatgaacac 1560

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ccatgacatc tgtggaccaa acaaggagct ggatgaagag acctgtcagt gtgtctgcag 1920

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<211> 2240
<212> DNA
<213> Mus musculus

<400> 18
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ccgttctcag tgccacaaat ttggtgccaa attctctcca gagaagcctc tctggaaact 420

tcccagagga tcccattcac cccagggccc tagctcctga tgactgcaga tcagacaagg 480

gctcagataa gcatactccc cccccccgt aacccctcc ccacatataa acctacagtt 540

atgcttccga ggtcaaacac gcaacttttt gggtgtgtgt gtatgtcaga aacacgcaat 600

tatttgggag ctcaaagtct gccgcactca agaatcatct ctcacccct ttccaagacc 660

cgtgccattt gagcaagagt tggggtgtgc ataatgtagt cactaggggg cgctcggcca 720

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